

Control of HslUV Protease Function by Nucleotide Binding and Hydrolysis

by

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B.S. Biochemistry
North Carolina State University, 2002

*Submitted to the Department of Biology in partial fulfillment
of the requirements for the degree of*

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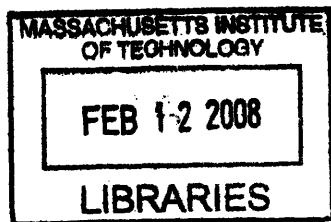
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ABSTRACT

Many proteins act as molecular machines, using the power of nucleotide binding and hydrolysis to drive conformational changes in themselves and their target substrates. Like other AAA⁺ proteases, HslUV recognizes, unfolds, translocates, and degrades substrate proteins in an ATP-dependent manner. Understanding how nucleotides interact with HslU and control the activities of both HslU and HslV provides insights into the general mechanism of energy-dependent proteolysis.

In order to better understand HslU-nucleotide interactions, I created a variant of HslU unable to hydrolyze ATP. HslU is composed of six identical subunits with a total of six nucleotide-binding sites. Moreover, many crystal structures show HslU with six bound nucleotides. Nevertheless, I found that HslU in solution is only able to bind 3-4 ATPs at saturation. This result rules out a model of ATP hydrolysis in which six nucleotides bind and are hydrolyzed together in a single power stroke and also suggests that many HslU crystal structures represent states that are not populated in the normal ATPase cycle. I also characterized the nucleotide requirement for various HslU activities. I found that at least two ATPs must be bound to HslU to support substrate binding and ATP hydrolysis, but showed that a single nucleotide is sufficient to support HslU-HslV binding and to stimulate HslV peptidase activity. I also found that the nucleotide state of HslU affects its affinity to HslV, weakening it when some subunits have ADP or no nucleotide bound. This effect is offset by an increase in HslU-HslV affinity during substrate degradation. This work suggests a simple model in which binding of a single ATP to HslU drives HslV binding, with further ATP binding acting to stabilize an HslU conformation that can bind protein substrate, hydrolyze ATP, and support substrate unfolding, translocation, and degradation.

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CHAPTER ONE

The AAA+ proteases HslUV, ClpXP, and the proteasome

Introduction

All organisms expend energy to grow, reproduce, and react to their environment. At the cellular level, the energy from nucleotide triphosphate (NTP) binding and/or hydrolysis is transduced into physical or chemical work. Specifically, hydrolysis of NTPs (typically ATP or GTP) is a highly favorable downhill reaction from a thermodynamic perspective. Macromolecular enzymatic machines are able to harness the energy of repetitive NTP binding and hydrolysis events to drive cyclical changes in three-dimensional structure and to transmit these conformational changes to bound macromolecules. During DNA replication, for example, ATP binding and hydrolysis by the clamp loader complex is coupled to conformational changes in the β or PCNA clamp proteins that close the clamp ring around DNA (Naktinis et al., 1995; Baker and Bell, 1998).

In the most common class of NTPases, two conserved sequence motifs play critical roles in NTP binding and hydrolysis. The Walker-A or P-loop motif (GX₄GKS/T) stabilizes binding through contacts with the nucleotide phosphates, whereas the Walker-B motif (Φ ₄DE; Φ = hydrophobic) is important for nucleotide hydrolysis (Hanson and Whiteheart, 2005). The AAA subfamily (ATPases associated with various cellular activities) is defined by a 200-250 residue ATPase domain, as well as a second region of homology (SRH) located C-terminal from the Walker-B motif. Analysis of multiple sequence alignments revealed additional related ATPases lacking the SRH, which comprise a AAA⁺ subfamily (Neuwald et al., 1999). AAA⁺ family members are involved in a wide range of cellular activities, including membrane fusion, transport,

protein disaggregation, transcription, DNA replication, DNA recombination, and proteolysis.

ATP-dependent proteolysis is a critical activity necessary for a variety of physiological functions and is carefully regulated so that only a specific set of proteins in the cell are subject to degradation at given time. There are many benefits of targeted degradation, including the removal of unwanted or deleterious proteins from the cell. Cellular levels of specific proteins vary depending on the environment. For example, under periods of stress (*e.g.*, heat shock, starvation, acid or oxidative stress, *etc.*), existing cellular proteins are often damaged as a consequence of unfolding, aggregation, chemical modification, or incomplete biosynthesis. If they cannot be repaired, these non-functional and potentially dangerous proteins are generally degraded. Moreover, during stress, transcriptional responses result in the synthesis of new and different types of proteins that allow the cell to deal with the specific stress and to minimize disruption. Once the environmental stress ceases, however, the proteome must be rebalanced through changes in transcription and proteolysis in order to return cellular processes and physiology to a normal state. During normal growth, proteolysis may also be used to maintain the concentration of certain proteins within a specific range or to release dormant activities through partial proteolysis. Thus, proteolysis provides a post-translational opportunity to regulate protein levels and activities in a variety of ways.

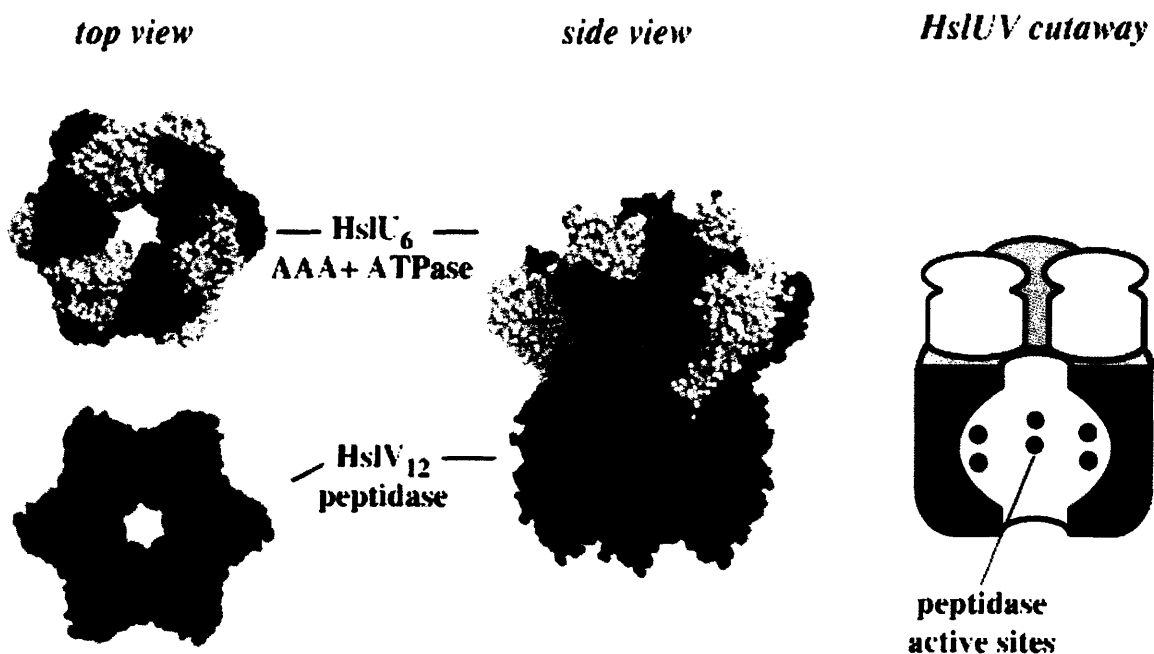
In *Escherichia coli* and related bacteria there are five distinct types of ATP-dependent AAA⁺ proteases: HslU/HslV (HslUV), ClpX/ClpP (ClpXP), ClpA/ClpP (ClpAP), Lon,

and FtsH (Neuwald et al., 1999; Gottesman, 1996). HslUV, ClpXP, and ClpAP are composed of separate AAA+ ATPase and peptidase components (Rohrwild et al., 1996; Katayama-Fujimura et al., 1987; Wojtkowiak et al., 1993). Lon and FtsH have both activities encoded on a single polypeptide chain (Charette et al., 1981; Tomoyasu et al., 1993). All of these enzymes are located in the cytoplasm, although FtsH is anchored to the inner side of the cytoplasmic membrane bound (Tomoyasu et al., 1993). Eukaryotes rely chiefly on the function of the 26S proteasome for degradation of cytoplasmic and nuclear proteins (Hershko and Ciechanover, 1998), although homologs of the bacterial AAA+ proteases carry out important degradation tasks within mitochondria and chloroplasts (Koppen and Langer, 2007). A closely related member of the AAA+ family powers each of these ATP-dependent proteases, and the distinct specificities of these enzymes allow cells to react to differential needs and to remodel the proteome in a dynamic fashion. In some cases, AAA+ ATPases function as disassembly chaperones to take apart macromolecular complexes and/or solubilize aggregates. For example, in the absence of protease partners, ClpX disassembles DNA-bound complexes of the MuA transposase and ClpB and Hsp104 resolubilize protein aggregates (Levchenko et al., 1995; Goloubinoff et al., 1999; Motohashi et al., 1999; Zolkiewski, 1999).

All AAA+ proteases share generally similar architectures. Stacking of hexameric or heptameric rings creates a hollow barrel-like structure in which the proteolytic active sites are hidden within an interior chamber to prevent accidental protein degradation. Hexameric AAA+ ATPases flank one or both sides of the protease barrel and function to recognize substrates, unfold native substrate structure, and translocate the unfolded

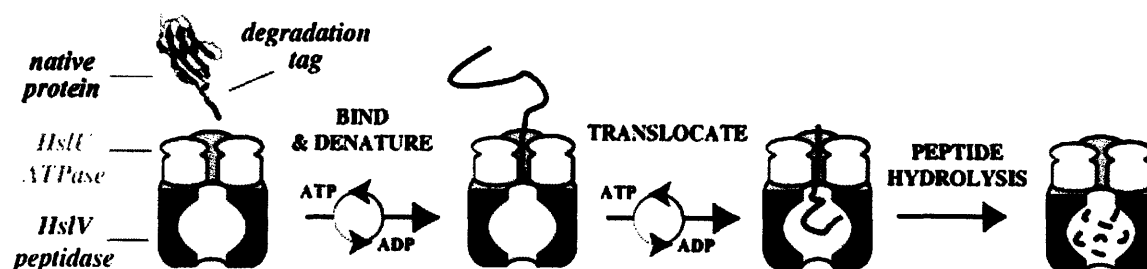
polypeptide into the protease chamber for degradation. Conserved AAA+ domains are responsible for these shared activities, whereas family specific domains are frequently implicated in substrate or adaptor recognition (Dougan et al., 2002; Mogk et al., 2004). The AAA+ module of each ATPase subunit is composed of a large $\alpha\beta$ domain and a smaller α -helical domain, with nucleotide binding occurring at subunit-subunit interfaces (Ogura and Wilkinson, 2001). Several highly conserved residues play key roles in nucleotide hydrolysis. Mutation of a highly conserved glutamic acid in the Walker-B motif is sufficient to disrupt ATP hydrolysis in many AAA+ proteins (Hersch et al., 2005; Hanson et al., 2005). In addition, highly conserved arginines play a critical role in sensing and hydrolyzing bound ATP and in mediating conformational changes that accompany binding and hydrolysis (Ogura et al., 2003).

Figure 1



The HslUV protease is composed of HslU and HslV (Figure 1). HslU is a homohexameric AAA+ ATPase, which adopts a ring-shaped structure with a central axial pore or channel. HslV, the peptidase component, is a threonine active-site protease related to the β subunits of the eukaryotic 20S proteasome. The functional HslV enzyme is assembled from 12 subunits, which form two stacked hexameric rings and enclose a degradation chamber in which the active-site threonines are located. This compartment or cavity is accessible through narrow axial pores. HslU hexamers can bind to one or both faces of HslV, resulting in alignment of their respective axial pores and the creation of a narrow channel into the lumen of the protease. HslU is responsible for recognizing appropriate protein substrates, unfolding them if necessary, and then translocating the denatured polypeptide into HslV for degradation (Figure 2). The unfolding and translocation steps require ATP hydrolysis, whereas peptide-bond cleavage by HslV does not.

Figure 2



From a structural perspective, HslUV is the best-characterized AAA+ protease. For example, there are more than 10 crystal structures of the HslU hexamer. Some structures contain isolated HslU hexamers; others contain complexes of one or two HslU hexamers

bound to HslV (Trame and McKay, 2001; Sousa et al., 2000; Wang et al., 2001a; Song et al., 2000; 2003; Bochtler et al., 2000). In some HslUV complexes, the active-site residues of HslV are chemically modified (Sousa et al., 2002; Kwon et al., 2003). The number of nucleotides bound to HslU varies from structure to structure. In some structures, for example, six ATP or ADP molecules are bound (Sousa et al., 2000; Kwon et al., 2003; Wang et al., 2001a; Song et al., 2000; Trame and McKay, 2001). In other crystal structures, only three or four nucleotides bind to the HslU hexamer (Bochtler et al., 1997; 2000). Although the abundance of structural information has allowed modeling of different conformations that may comprise the ATPase cycle (Wang et al., 2001a), it is still unclear which, if any, of the available crystal structures represent functional states of the HslUV protease. My aim in this thesis is to characterize HslUV from a biochemical perspective, thereby providing a complement to the structural information and improving our understanding of HslUV mechanism.

In the sections below, I provide an introduction to HslUV, related proteases, and their interaction with substrates and nucleotides. Although much is known about ATP-dependent proteolysis for enzymes like ClpXP and ClpAP, there have been far fewer studies of HslUV. HslV is the bacterial homolog of the eukaryotic 20S proteasome, which is an attractive pharmaceutical target given its roles in a variety of pathways, including inflammation and differentiation. With only two types of subunits, HslUV is far simpler and more accessible to dissection than the 26S proteasome, which contains more than 20 types of subunits, and yet both enzymes perform a core set of common functions. Studies of the detailed mechanisms used by HslUV to degrade substrates also facilitates

comparisons with better characterized AAA+ proteases, such as ClpXP, and should provide insights into common functions and family-specific features. When I began my thesis work, it was known that ATP was needed for many functions of HslUV. ATP or ATP γ S was required for activation of HslV and for interactions between HslU and HslV (Huang and Goldberg, 1997; Yoo et al., 1997a; Burton et al., 1995). However, specific roles were unclear. How many nucleotides HslU was capable of binding, whether there were single or multiple classes of nucleotide binding sites, and how nucleotide binding was linked to specific HslUV activities were all undefined. The importance of nucleotides in AAA+ protease complex formation and substrate recognition has been documented in other systems (Bolon et al., 2004; Smith et al., 2005). Because ATP fuels the HslUV machine, determining how nucleotide binding and hydrolysis in HslU control interactions with substrate and HslV is important for understanding function.

HslUV Characterization

The HslUV protease is present in roughly 60% of eubacteria and many eukaryotic lineages (Gille et al., 2003; Ruiz-Gonzales and Marin, 2006). The tandem genes encoding HslU (also called ClpY) and HslV (also called ClpQ) were originally discovered by DNA sequencing and were found to be under the control of a heat-shock promoter (Chuang and Blattner, 1993). The names stand for heat shock locus proteins U and V. These genes and/or the encoded enzymes were also identified in other contexts (Missiakas et al., 1996; Khattar, 1997). Early studies on the physiological role of HslUV found that *E. coli* knockout strains were viable and showed no obvious phenotypic effects below 45 °C; above this temperature, deletion strains grew slowly or died (Katayama et al., 1996

Kanemori et al., 1997). Other heat-shock proteins were found to be overproduced in the knockout strains, and overexpression of HslUV reduced the heat-shock response (Missiakas et al., 1996). The purified HslUV protease from *E. coli* shows maximal degradation activity between 50 and 60 °C, a temperature range in which some AAA+ proteases, like ClpXP, denature and lose all activity (Burton et al., 2005). As a consequence, it seems likely that HslUV plays a special role in degrading cellular proteins that unfold or become damaged at high temperatures. Indeed, HslUV was shown to participate in degradation of incomplete and thus presumably unfolded proteins produced by premature puromycin-induced termination of translation (Missiakas et al., 1996). The presence of unfolded proteins in *E. coli* induces the heat-shock response (Parsell and Sauer, 1989). Thus, the increased levels of heat-shock proteins in strains lacking HslUV can be explained by over accumulation of denatured proteins, whereas HslUV overexpression would be expected to reduce the concentration of unfolded proteins and therefore diminish the heat-shock response. HslU has also been shown to play a role in the disaggregation of proteins, an activity shared by related AAA+ ATPases (Seong et al., 2000).

In addition to proteins damaged by thermal denaturation, several other physiological substrates of HslUV have been identified. The heat-shock transcription factor σ^{32} , which is responsible for the expression of HslUV (Rohrwild et al., 1996), is degraded primarily by FtsH, but also by HslUV and to lesser extents by Lon and ClpXP (Kanemori et al., 1997). RcsA (the capsule-synthesis activator) and Sula (a cell-division inhibitor) are also substrates for HslUV and for Lon (Wu et al., 1999). For example, overexpression of

HslUV suppresses phenotypes caused by stabilization of both proteins in Lon-knockout strains (Kuo et al., 2004). Pulse-chase analysis showed that HslUV degradation of Sula occurs in the absence of heat shock, and thus HslUV presumably also plays roles in regulation and protein-quality control under normal physiological conditions (Seong et al., 1999). HslUV and Lon are both required for cell viability under conditions in which the signal recognition particle (SRP) is expressed at low levels. SRP is responsible for proper cotranslational insertion of proteins into the inner membrane of *E. coli*. When SRP activity is limited, inner-membrane proteins accumulate in the cytoplasm, suggesting that both HslUV and Lon function to remove these otherwise toxic mislocalized proteins (Bernstein and Hyndman, 2001). Taken together, these results indicate that HslUV degrades numerous intracellular substrates, often sharing overlapping functions with other ATP-dependent proteases. Removal of many of these substrates seems to be sufficiently important that multiple redundant pathways have evolved to ensure degradation.

In general, known physiological substrates of HslUV have not been very useful as model substrates for studies *in vitro*. For example, Sula and σ^{32} are both relatively difficult to express, are poorly soluble, and exhibit short intracellular half-lives under normal conditions (Nguyen et al., 1993; Kwon et al., 2004). There is little structural information for either protein (Kwon et al., 2004). Sula has been purified as a fusion protein to maltose-binding protein (MBP) and green fluorescent protein (GFP), but HslUV is incapable of degrading either the MBP or GFP portions of these fusions (Seong et al., 1999; Kwon et al., 2004). This result suggests that HslUV may not have a highly robust

unfoldase activity. The HslUV cleavage sites in Sula have been mapped by analysis of peptide products, showing that HslV prefers to cleave after hydrophobic residues (Nishii and Takahashi, 2003). However, HslUV degrades Sula very slowly *in vitro*, and thus non-physiological substrates have typically been used for detailed biochemical studies.

In early studies, the proteolytic activity of HslUV was usually monitored by degradation of ^3H - or ^{14}C -labeled casein or insulin B-chain (Huang and Goldberg, 1997; Seol et al., 1997). These generic substrates have no stable tertiary structure and are degraded by most proteases, but are only bound weakly by HslUV and are degraded at slow rates. Moreover, HslUV degradation of casein does not require ATP (Huang and Goldberg, 1997). This result suggests that casein does not have to be actively translocated through HslU and thus does not serve as a good model for normal degradation.

More recently, it has been found that HslUV efficiently degrades the Arc repressor from bacteriophage P22 (Kwon et al., 2004; Burton et al. 2005). The structure of Arc, a dimeric ribbon-helix-helix transcription factor, is known and its stability to denaturation has been well characterized (Breg et al., 1990; Sauer et al., 1996). Steady-state kinetic parameters have been determined for HslUV degradation of Arc mutants of various stabilities, as well as for variants fused to the I27 domain of human titin, a protein that is very resistant to mechanical unfolding (Carrion-Vazquez et al., 1999; Burton et al., 2005). Stable Arc substrates are recognized and degraded efficiently ($K_m \sim 5 \mu\text{M}$; turnover number $\sim 7 \text{ min}^{-1}$). The titin domain fused to Arc is also degraded. Thus, HslUV is able to unfold substrates that are quite stable prior to degradation. HslUV recognizes

residues at both the N- and C-terminus terminus of Arc (Kwon et al., 2004; Burton et al. 2005; Sundar and McGinness, personal communication). Based on the N-terminal sequence of Arc, peptide-array experiments have been used to identify a peptide (called gt1) that binds HslU in a nucleotide-dependent fashion and competes with binding and degradation of Arc (Burton et al., 2005).

When HslUV was initially purified, a variety of fluorogenic peptides were screened as potential substrates (Rohrwild et al., 1996). N- α -benzyloxycarbonyl-Gly-Gly-Leu-7-amido-4-methylcoumarin (Z-GGL-AMC), a substrate previously used to assay the chymotrypsin-like activity of the proteasome (Chu-Ping et al., 1992), was found to be cleaved efficiently by HslUV (Rohrwild et al., 1996). In the absence of HslU, HslV degrades Z-GGL-AMC very slowly, and this activity is inhibited by nucleotide (Huang and Goldberg, 1997; Lee et al., 2007). The physiological relevance of the latter observation is unclear. In the presence of HslU and a suitable nucleotide triphosphate (ATP, AMPPNP, or ATP γ S), however, HslV cleavage of Z-GGL-AMC is stimulated roughly 500-fold (Huang and Goldberg, 1997). Thus, by itself, HslV is a poor peptidase, degrading even small peptides far more slowly in the absence than the presence of HslU. Because the axial pore of HslV is large enough to allow Z-GGL-AMC to diffuse into the peptidase chamber, the active sites of this enzyme must be largely unreactive when HslU is not bound. Because HslU in combination with ATP or the poorly hydrolyzed ATP γ S analog or the non hydrolyzable AMPPNP analog stimulates the peptidase activity of HslV to comparable extents, NTP binding but not hydrolysis seems to be required to allow HslU to interact productively with HslV. As discussed below, NTP binding to

HslU appears to place its C-terminal tail in a conformation that binds and activates HslV (Seong et al., 2002; Sousa et al., 2002). In Chapter 2, I use nucleotide-dependent activation of HslUV cleavage of Z-GGL-AMC to probe the strength and requirements of HslU binding to HslV.

HslU and HslUV Crystal Structures

Like all AAA+ ATPases, HslU subunits contain a large and a small AAA+ domain, and nucleotides bind at the interface formed by both of these domains in a single subunit and by the large AAA+ domain of an adjacent subunit (Bochtler et al., 2000; Sousa et al., 2000). This architecture allows changes in nucleotide occupancy and/or identity to alter the tertiary and quaternary structure of the HslU hexamer, giving rise to conformational transitions during the ATPase cycle that presumably explain its machine-like properties. Indeed, the orientations of the large and small AAA+ domains vary in different HslU structures and do seem to depend upon nucleotide state; these observations have been used to propose a series of structural changes that occur during the nucleotide hydrolysis cycle (Wang et al., 2001a). As I discuss below, however, it is unclear how many of the subunit conformations observed in different HslU crystal structures are functionally relevant.

E. coli HslU and its orthologs contain an additional “intermediate” or I-domain. If HslU is viewed as having the basic shape of a hexagonal nut, then the I-domain projects away from one face of the nut and HslV binds to the other face (Bochtler et al., 2000; Sousa et al., 2000). Thus, the I-domain is on the face of HslU where substrates are thought to bind

initially. HslU variants in which the I-domain is deleted cannot support HslV degradation of Sula or Arc repressor but some casein degradation is observed (Song et al., 2000; Kwon et al., 2003). Thus, the I-domain probably plays a role in the recognition of specific protein substrates.

Table 1

Organism	Protein	HslU Tail Conformation	Year	Nucleotide State	PDB Accession
<i>H. influenzae</i>	UV	extended	2000	6 ATP	1G3I
<i>H. influenzae</i>	U(Δ I)V	extended	2003	6 ADP (Pi, Mg ⁺⁺)	1OFH
<i>E. coli</i>	UV	packed	2001	6 dATP	1G4A
<i>E. coli</i>	UV	packed	2000	6 AMPPNP	1E94
<i>E. coli</i>	U	packed	2000	4 ATP	1DO0
<i>H. influenzae</i>	U	packed	2001	6 ADP	1G41
<i>E. coli</i>	U	packed	1997	3 AMPPNP, 3 empty	1D02
<i>H. influenzae</i>	U	packed	2001	6 ADP	1IM2
<i>E. coli</i>	UV	packed	2000	6 ADP	1HQY
<i>E. coli</i>	UV	packed	2000	6 ADP	1HT1
<i>E. coli</i>	UV	packed	2000	6 ADP	1HT2
<i>E. coli</i>	UV	packed	2000	none	1G4B

HslUV is the only ATP-dependent protease for which the complete three-dimensional structure has been solved. Crystal structures of HslU₆, HslV₁₂, HslU₆HslV₁₂, and HslU₆HslV₁₂HslU₆ are known (Bochtler et al., 1999; Sousa et al., 2000; 2002; Sousa and McKay, 2001; Trame and McKay, 2001; Wang et al., 2001a; Kwon et al., 2003; Song et al., 2003). Table 1 lists the types and numbers of nucleotides bound to HslU in the available structures. Many of the known HslU structures are six-fold symmetric and have ATP or ADP bound to each potential nucleotide binding site, as would be expected for a model in which HslU cycled between an all-ATP state and an all-ADP state in a concerted allosteric reaction. However, one HslU structure is a trimer of dimers with AMPPNPs bound to alternating subunits, and another is a dimer of trimers with adjacent subunits in each trimer being nucleotide-free, ATP-bound, and ATP•Mg⁺⁺-bound,

respectively. These non-symmetric structures suggest that HslU may operate by some type of sequential mechanism in which only a subset of bound nucleotides can be hydrolyzed.

In some cases, identification of the nucleotides bound in HslU structures has been a matter of dispute. For example, one HslU structure (1DOO) with six putatively bound AMPPNPs was found to have a structure indistinguishable from another structure (1G4A) in which six ADPs were bound (Bochtler et al., 2000; Song et al., 2000; Wang et al., 2001a; 2001b). When the 1DOO structure was recalculated, however, no electron density was observed for the γ -phosphate of AMPPNP (Wang et al., 2001b). Additionally, the adenine base in the 1DOO structure was built in a *syn* conformation, whereas most nucleotides bound to AAA+ ATPases adopt an *anti* conformation (Wang et al., 2001b). In this instance, it seems clear that the AMPPNP in the 1DOO structure was misidentified and is actually ADP, presumably present as a contaminant in the AMPPNP or enzyme used for crystallization. Indeed, in another HslU structure (1DO0), nucleotides were found bound to HslU even though nucleotide had not been included in the crystallization drop. Because of these issues, the identity of nucleotides in other HslU structures has also been questioned. For example, in both asymmetric HslU structures, the adenines of ATP (1DO0) or AMPPNP (1DO2) were *syn* and the temperature factors of the γ -phosphate atoms were roughly 50 Å² higher than those of surrounding atoms, raising the possibility of misidentification.

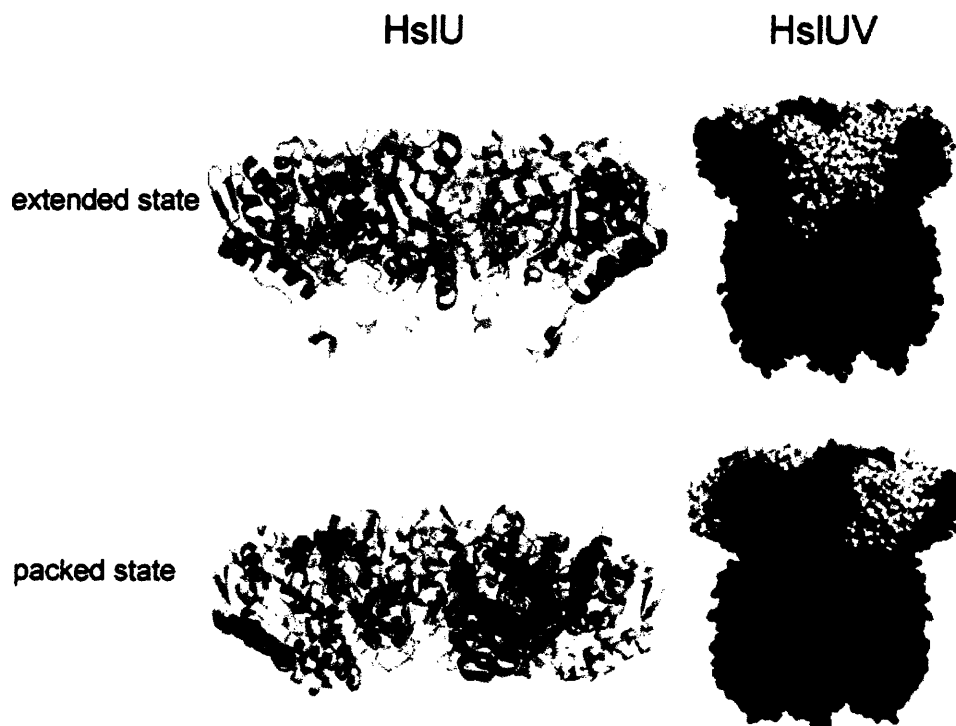
HslV

As noted above, the peptidase active sites, including the catalytic N-terminal threonine, are sequestered in an internal cavity in the double-ring HslV dodecamer. HslV has the distinct fold shared by members of the N-terminal nucleophile (Ntn) hydrolase family. Originating from a common ancestor, the sequences of family members, which have a variety of hydrolytic activities, are now highly diverged. Other members of this family include penicillin acylases, glutamine PRPP amidotransferases, glycosylasparaginases, and notably the 20S proteasome (Brannigan et al., 1995). In the proteasome, autocatalytic processing reveals the N-terminal catalytic threonine; in HslV, removal of the initiator formyl methionine by the combined action of peptide deformylase and methionine amino-peptidase frees threonine as the N-terminal residue. Comparison of HslV structural data suggests that a nearby lysine in the active site lowers the pK_a of the α -amino group of Thr¹, which in an unprotonated state can accept a proton from and activate the Thr¹ side chain for nucleophilic attack (Groll et al., 1995). Replacing Thr¹ in *E. coli* HslV with a serine results in peptidase levels similar to wild type when stimulated by HslU (Yoo et al., 1997b). In fact, the *B. subtilis* HslV homolog, CodW, is the only known N-terminal serine protease. Serine-protease inhibitors inactivate CodW, and replacing Ser¹ with threonine abolishes all enzymatic activity (Kang et al., 2001).

Comparison of HslV structures in combination with biochemical results has provided key insights into the mechanism of HslV activation by HslU. HslV structures from *E. coli*, *Thermatoga maritima*, and *Haemophilus influenzae* (Bochtler et al., 1997; Song et al., 2003; Sousa and McKay, 2001) have been solved. The structure of *H. influenzae* HslV

has the highest resolution (2.1 Å), and this enzyme has been crystallized in complex with HslU (Sousa and McKay, 2001). The structures of free and HslU-bound HslV reveal conformational differences, as expected from the finding that the active sites of HslU-bound HslV react with vinyl sulfone inhibitors, whereas the active sites of free HslV do not (Bogyo et al., 1997). Moreover, when inhibitor was soaked into crystals containing a single HslU₆ bound to HslV₁₂, the active sites in the *cis*-HslV ring but not the *trans*-HslV ring were modified (Kwon et al., 2003). These results suggest that HslU binding is required to stabilize an HslV conformation in which the active-site residues assume the correct catalytic geometry and that an HslU hexamer only activates the HslV ring to which it binds. This model explains why HslUV but not HslV cleaves the Z-GGL-AMC peptide efficiently.

Figure 3



In several *H. influenzae* HslUV structures, the C-terminal tails of HslU form an extended conformation and pack in grooves between HslV subunits (Trame and McKay, 2001; Figure 3; upper panel). These interactions cause changes in the conformation of the apical helices of HslV, which are propagated to the active site (Sousa et al., 2002). In structures of the *E. coli* HslUV complex, by contrast, the C-terminal tails of HslU pack back against structural elements in HslU (Figure 3, lower panel). Mutational and peptide experiments support the importance of the C-terminal peptides of HslU in activation of HslV. Specifically, mutations that increased the size of the HslV pore did not stimulate peptidase activity in the absence of HslU, whereas deletion and point mutations in the C-terminal residues of HslU prevented HslV activation (Ramachandran et al., 2002; Seong et al., 2002). Moreover, high concentrations of synthetic peptides corresponding to just the 8 C-terminal residues of *E. coli* HslU were shown to activate HslV cleavage of Z-GGL-AMC substantially (Ramachandran et al., 2002). These results raise the question of why the C-terminal peptides of HslU are extended to interact with HslV in some structures but not in others. One likely possibility is that identity of bound nucleotides determines whether the C-terminal tails are extended. This model is consistent with the observation that HslU•ADP does not activate HslV, whereas HslU•ATP, HslU•ATP γ S, and HslU•AMPPNP do activate HslV.

The ClpXP system

ClpX shares significant sequence and structural homology with HslU, and the ClpXP system has been characterized extensively as a model for ATP-dependent protein

degradation. ClpX is a homohexameric AAA+ ATPase, which binds to one or both sides of ClpP, a double-ring tetradecameric serine peptidase. Like HslUV and most other ATP-dependent proteases, the architecture of ClpXP resembles an extended barrel with the proteolytic chamber of ClpP in the center. A narrow axial channel, through ClpP and ClpX, connects the protease chamber with the outside world.

The protein substrates of ClpXP are recognized via short peptide tag sequences, usually located at the N- or C-termini. For example, ClpX recognizes –Leu-Ala-Ala-COO⁻ at the C-terminus of ssrA-tagged proteins (Gottesman et al., 1998; Flynn et al., 2001) but also recognizes different sequence motifs at both N- and C-terminal positions of natural substrates (Flynn et al., 2003). Recognition of ssrA-tagged substrates requires an ATP-bound form of ClpX (Bolon et al., 2004; Hersch et al., 2005). ATP binding is also needed for HslU recognition of some protein substrates (Burton et al., 2005). Some ClpXP substrates are delivered by adaptor proteins, a theme that is common for AAA+ enzymes (Baker and Sauer, 2006). For example, the SspB adaptor binds both to ClpX and to ssrA-tagged substrates, facilitating tethering of these substrates to ClpXP (Levchenko et al., 2000; Wah et al., 2003). SspB-mediated tethering lowers K_M for ClpXP degradation of ssrA substrates about 10-fold, but modifications of the ssrA tag that reduce ClpX binding result in much larger effects and make substrate binding effectively adaptor-dependent (McGinness et al., 2006; 2007).

Studies of protein degradation by ClpXP have led to models in which translocation of a substrate peptide tag through the narrow central channel results in the application of a

mechanical unfolding force when a folded substrate cannot fit through this pore (Kim et al., 2000; Kenniston et al., 2003). An alternative model in which ClpX simply traps globally unfolded substrates have been ruled out because ClpXP degradation of certain substrates, like GFP, occurs more than 10^6 -fold faster than the solution-unfolding rate (Kim et al., 2000). The resistance of proteins to ClpX-induced unfolding and degradation can vary substantially and appears to correlate with the local stability of the protein region that bears the degradation tag rather than with global protein stability (Lee et al., 2001; Kenniston et al., 2003; 2004). If a native protein resists ATP-dependent unfolding by ClpXP, it is released from the enzyme and must rebind before another unfolding attempt can be made (Kenniston et al., 2005). This feature allows ClpXP to degrade the most susceptible substrates available and prevents trapping of the enzyme in futile unfolding attempts, but it also has a high energetic cost. For example, ClpXP may need to hydrolyze an average of more than 500 ATPs to ensure degradation of a single substrate that resists unfolding (Kenniston et al., 2003).

Detailed studies of ATP binding to ClpX have shown that it only binds 3-4 nucleotides at saturation and support the existence of three classes of ClpX subunits in active hexamers (Hersch et al., 2005). The N- and C-termini of ClpX subunits can also be connected by flexible linkers, allowing biochemical studies of “single-chain” hexamers (Martin et al., 2005). In these covalent hexamers, individual subunits can be independently mutated, allowing determination of the number of active subunits needed for ClpX function. A single-chain ClpX hexamer with one wild-type subunit and five subunits incapable of hydrolyzing ATP supported low levels of ClpXP degradation, indicating that the power

stroke can arise from hydrolysis in a single subunit. In addition, covalent hexamers composed of two wild-type subunits, two subunits that mimic nucleotide-free subunits, and two subunits that could bind but not hydrolyze ATP were found to degrade substrates with the same energetic efficiency as wild-type ClpXP. These studies support the idea that individual subunits in a ClpX homoheptamer assume distinct functional roles, and are inconsistent with models that require concerted ATP hydrolysis or a strict pattern of sequential hydrolysis. One model, consistent with the available information, is that that ATP hydrolysis is sequential but probabilistic (Martin et al., 2005). In such a model, following ATP hydrolysis in one subunit, any of the remaining ATP-bound subunits could be the next to fire, potentially depending of which are stimulated by contact with a translocating substrate.

The 26S Proteasome

Because HslV is related to the β -subunits of the eukaryotic 20S proteasome, it has been considered to be the bacterial homolog. The 20S proteasome is a barrel-shaped protease, also called the core particle (CP), with a mass of approximately 700 kDa. Together with the 19S regulatory complex, it is responsible for most degradation of cytosolic and nuclear proteins. The 20S particle is composed of stacked rings: two β_7 rings form the core, which is then flanked by two α_7 rings. This architecture creates three chambers: a large central chamber created by the β rings, in which hydrolysis of peptide bonds occurs, and two smaller chambers created at the α/β interfaces. Both the α and β subunits have diverged into unique sequences. The β subunits provide the proteolytic activity, although only three of the seven subunits in a ring (β_1 , β_2 , and β_5) are active. These subunits

provide a trypsin-like, a chymotrypsin-like, and a caspase-like peptidase activity (Hershko and Ciechanover, 1998). Two additional activities, a preference to cleave after branched amino acids and after small neutral amino acids, are shared among the three active β subunits (Groll et al., 2005).

The α subunits have a fold similar to the β subunits, but their function seems to be to restrict access to the active sites in the β subunits instead of providing peptidase activity. The N-termini of the α subunits cover the top of the axial pore in an asymmetric overlapping manner, thereby physically occluding entry into the CP (Groll et al., 1997). Restructuring of the α N-termini, which occurs upon binding of regulatory proteins, causes these tails to extend distally and allows access to the proteolytic sites. Thus, activation allows enhanced peptidase, protease, or ubiquitin-protein degradation (Bajorek and Glickman, 2004). Deletion of just 9 N-terminal residues of α_3 ($\alpha_3\Delta N$) results in peptidase activation equivalent to or greater than observed for the stimulated wild-type enzyme (Groll et al., 2000; Kohler et al., 2001). Interestingly, these N-terminal sequences can also be added *in trans* to block access (Groll et al., 2000). SDS also stimulates CP peptidase activity (Dahlmann et al., 1985), presumably by stabilizing the open conformation.

Gate opening caused by restructuring of the N-terminal tails of the α subunits is normally induced by binding of either the 19S (PA700) regulatory particle (RP) or the 11S (PA28) regulatory complex. PA28 functions to increase production of small peptides for display by MHC class I molecules (Groettrup et al., 1996; Dick et al., 1996). However, the

mechanism of gate opening appears to be different for the 19S and PA28 complexes, as there is little sequence or structural similarity between these complexes. The base of the RP is composed of six homologous ATPase subunits, Rpt1-6 (Glickman et al., 1998; Fu et al., 2001). This base complex is responsible for gate-opening activity and also plays roles in unfolding and translocation. PAN, the archaeal homolog of the base complex, PA26, the archaeal homolog of PA28, and the archaeal 20S peptidase provide a simpler but related model system. The archaeal 20S peptidase is composed of single types of α and β subunits, and the PAN/20S enzymes are easily purified and do not require ubiquitin-conjugated proteins for degradation (Smith et al., 2007). By extending into pockets between the α subunits, the C-terminal tails of PA700/PAN or PA28/PA26 play important roles in docking with and activating the CP (Whitby et al., 2000, Smith et al., 2007).

Gate opening by the PA26 heptamer requires interactions between the C-terminal tails of PA26 and the 20S particle, and also requires a contact made by conserved lysine in an activation domain located distantly from the PA26 C-termini (Zhang et al., 1998; Forster et al., 2005). In contrast, peptides corresponding to the PAN C-terminal tails can activate 20S gate opening in isolation, supporting a sufficient role in the PAN complex, although only specific Rpt tails stimulate comparable gate opening in mammalian 20S proteasomes (Smith et al., 2007). Unlike PA26/PA28, the base complex of the 19S regulatory particle and PAN are hexameric and thus are mismatched with the 7-fold symmetric 20S particle. Geometric constraints suggest that only a subset of PAN or base complex tails actually interact with the 20S complex, and thus a full set of six interactions

may not be required for gate opening (Smith et al., 2007). However, binding of the ClpX hexamer to a 7-fold symmetric ClpP ring also results in a symmetry mismatch, but recent studies show that six loops from ClpX must all contact ClpP for tight binding (Martin et al., 2007).

There are both similarities and differences between activation of HslV and the 20S proteasome. In both cases, the C-terminal tails of a binding partner play important roles in activation. HslU, like 19S/PAN, provides ATPase activities required for unfolding and translocation of substrates. However, there is no mismatch in binding of HslU hexamers to the 6-fold symmetric rings of HslV₁₂. Additionally, HslV activation is not caused by gate opening, but rather by conformational changes induced by the binding of HslU or its C-terminal peptides. Nevertheless, it is intriguing that some aspects of the activation mechanism have been conserved for these two distantly related proteolytic systems.

Research Approach

In this introductory chapter, I have discussed HslUV and related ATP-dependent protein degradation machines. Many of the molecular mechanisms by which these complexes interact with and degrade substrates are probably shared. Thus, studies of individual systems have the potential to illuminate common themes of protein degradation or to identify family specific activities and regulatory strategies. Understanding how these proteases interact with and utilize ATP is central to understanding their roles as ATP-powered machines. In chapter 2, I characterize nucleotide interactions with HslUV and describe how HslUV activities are regulated by the nucleotide state of HslU. Using both

wild-type HslU and a mutant defective in ATP hydrolysis, I was able to assay nucleotide binding and its linkage to coupled activities such as substrate recognition and HslV activation. I found that HslU binds a maximum of 3-4 nucleotides, suggesting the majority of HslU crystal structures do not represent functional states of the enzyme. In chapter 3, I characterize HslU mutants with alterations in the nucleotide-binding sites or in conserved pore loops to clarify how these structures facilitate substrate binding and HslV activation. In chapter 4, I discuss unsuccessful efforts to establish a solution-based physical binding assay between HslU and HslV using fluorescence resonance energy transfer (FRET).

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Asymmetric nucleotide transactions of the HslUV protease

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Abstract

ATP binding and hydrolysis are critical for protein degradation by HslUV, a AAA+ machine containing one or two HslU₆ ATPases and the HslV₁₂ peptidase. Although each HslU homohexamer has six potential ATP-binding sites, we show that only three or four ATP molecules bind at saturation and present evidence for three functional subunit classes. Our results support an asymmetric mechanism of ATP binding and hydrolysis and suggest that molecular contacts between HslU and HslV vary dynamically throughout the ATPase cycle. Nucleotide binding prioritizes HslUV assembly and activity. For example, binding of a single ATP stabilizes HslU binding to and activation of HslV, whereas binding of multiple ATPs is required for protein-substrate recognition, for ATP hydrolysis, and for subsequent substrate unfolding and translocation. Thus, a simple thermodynamic hierarchy ensures that substrates bind to functional HslUV complexes and that ATP hydrolysis is efficiently coupled to protein degradation. Moreover, the differential effects of ATP binding on HslU activity provide a fail-safe mechanism that ensures highly processive protein degradation.

AAA+ protease/ energy-dependent degradation/ protein turnover/ enzyme regulation/
peptidase activation/ HslVU

Introduction

ATP fuels the operation of most molecular machines, including energy-dependent intracellular proteases. ATP-dependent proteases typically consist of a barrel-shaped peptidase, with the active sites for peptide-bond cleavage sequestered in an aqueous internal chamber, and associated hexameric AAA+ ATPases that recognize specific protein substrates, unfold these molecules, and then translocate the denatured polypeptide into the peptidase chamber for degradation (Pickart and Cohen, 2004; Sauer et al, 2004). AAA+ and related P-loop ATPases also operate in many other biological processes in which mechanical work is performed on macromolecules (Neuwald et al, 1999; Hanson and Whiteheart, 2005).

HslUV is an ATP-dependent protease present in roughly 60% of eubacteria and many eukaryotic lineages (Ruiz-Gonzales and Marin, 2006). Functional HslUV enzymes are produced by the binding of one or two AAA+ HslU₆ hexamers to HslV₁₂, a double-ring dodecameric peptidase that shares an N-terminal active-site threonine and extensive structural homology with the β -subunits of the eukaryotic proteasome (Rohrwild et al, 1996; Kessel et al, 1996; Bochtler et al, 1997). Importantly, crystal structures of HslU₆, HslV₁₂, HslU₆•HslV₁₂, and HslU₆•HslV₁₂•HslU₆ (Fig. 1A) have been solved (Bochtler et al, 1997; 2000; Song et al 2000; Sousa et al, 2000; 2002; Sousa and McKay, 2001; Trame and McKay, 2001; Wang et al, 2001a; Kwon et al, 2003; Song et al, 2003). Moreover, many HslU structures differ in conformation, apparently as a consequence of differences in bound nucleotides, providing clues about potential nucleotide-dependent movements that may drive protein unfolding and translocation. Indeed, HslUV is the only ATP-

dependent protease for which the entire structure is known. However, several issues have hampered further understanding of this enzyme. First, structures of HslU hexamers with six, four, or three bound nucleotides have been solved. Which of these structures are functionally relevant is unclear. Second, whether the nucleotide bound in different HslU crystal structures is ADP or ATP has been controversial (Wang et al, 2001b). Third, little quantitative information is available about nucleotide binding to HslU in solution or about the functional consequences of this binding on HslU recognition of HslV and/or protein substrates.

ClpX is a hexameric ATPase that shares about 50% sequence homology and substantial structural homology with HslU (Neuwald et al, 1999; Kim and Kim, 2003). Under conditions of ATP saturation, hexamers of ClpX bind only three or four molecules of ATP (Hersch et al, 2005). In addition, single-chain ClpX hexamers are functional with two subunits able to hydrolyze ATP, two hydrolytically inactive subunits capable of assuming an ATP-bound conformation, and two subunits that mimic ATP-free wild-type subunits (Martin et al, 2005). Studies of single-chain ClpX variants also suggest that hydrolysis is probabilistic in the sense that different subunits hydrolyze ATP in a sequential but not necessarily ordered fashion. If HslU and ClpX function in the same manner, then active HslU hexamers probably also operate sequentially, always containing a mixture of nucleotide-bound and nucleotide-free subunits. However, ClpX partners with ClpP, a peptidase that shares no structural homology with HslV (Wang et al, 1997), and thus it is not obvious that ClpX and HslU will operate by similar mechanisms. Indeed, structural studies of several other hexameric ATPases have been interpreted as

evidence that these enzymes cycle between states that are fully ATP-bound or ADP-bound (Gai et al, 2004; Chen et al, 2007; Lee et al, 2007). Moreover, most HslU and HslUV crystal structures have six bound ATPs or ADPs (Fig. 1B), apparently supporting a symmetric or concerted mechanism of ATP hydrolysis.

Here, we demonstrate that only three or four molecules of ATP bind to the HslU homo-hexamer at saturation. Thus some of the six potential binding sites in the hexamer remain empty. ATP binds the sites that can be filled with comparable affinities, although the kinetics of nucleotide dissociation reveals the existence of two classes of binding sites. Our results support a sequential ATPase model in which HslU subunits function asymmetrically and highlight structures with three or four bound nucleotides as being the most relevant for understanding HslU function. Importantly, we show the existence of a fail-safe mechanism in which binding of a single ATP supports HslV binding by the HslU hexamer, whereas multiple ATPs are required for nucleotide hydrolysis and protein-substrate binding. Finally, we present evidence that interactions with HslV vary as subunits of the HslU hexamer transit through the ATPase cycle.

Results

HslU hydrolyzes ATP rapidly, complicating studies of ATP binding using the wild-type enzyme. To avoid this problem, we mutated Glu²⁵⁷ in the Walker-B box of *Escherichia coli* HslU to Gln (E257Q). We anticipated that this mutant would be defective in ATP hydrolysis because the Glu side chain appears to be close to the γ phosphate of bound ATP, glutamate is invariant at this position in HslU orthologs, and the same mutation reduces ATP hydrolysis markedly in related AAA+ enzymes (Bochtler et al, 2000; Sousa

et al, 2000; Hersch et al, 2005; Hanson et al, 2005).

HslU E257Q with an N-terminal His₆ tag behaved similarly to His₆-tagged wild-type HslU during purification steps involving anion-exchange chromatography and gel filtration, suggesting that both proteins had similar structures. However, unlike wild-type HslU, the ultraviolet spectrum of the E257Q protein following these steps indicated the presence of bound nucleotide, which was not included in the purification. This observation suggested that nucleotide from the cell extract remained bound to the mutant, suggesting tighter binding than to wild-type HslU. Incubation with high salt, which dissociates the HslU hexamer (Song et al, 2000), and activated charcoal, which binds nucleotide, resulted in a spectrum of the mutant protein that was similar to that of wild-type HslU (Supplemental Figure 1). Moreover, in both cases, the spectra were those expected based on the number of tyrosines and tryptophans in these proteins. Treatment of wild-type HslU with charcoal and high salt did not affect its spectrum, ATP-hydrolysis activity, stimulation of HslV peptidase activity, or ability to support HslV degradation of Arc repressor, a good substrate (Supplemental Figures 2-4).

Compared to wild-type HslU, the E257Q mutant had almost no ATPase activity by itself, or when HslV or Arc repressor and HslV were added (Fig. 1C; Kwon et al, 2004; Burton et al, 2005). Indeed, ATP hydrolysis in reactions containing the E257Q mutant was similar to control reactions lacking this protein. Moreover, no degradation of Arc repressor was detected in the presence of HslV, E257Q HslU, and ATP (Supplemental Figure 5).

HslU hexamers bind three or four ATPs

Knowing how many ATPs bind to a HslU hexamer is important for understanding mechanism and for evaluating which crystal structures are likely to represent functional complexes. To determine stoichiometry, increasing amounts of ATP were added to a fixed concentration of the E257Q hexamer, and binding was assayed by isothermal titration calorimetry (ITC). As shown in Fig. 2A, saturation occurred at an ATP/hexamer ratio between three and four. Fitting of these data gave an average stoichiometry of 3.5 ATPs per hexamer ($\Delta H = -4.1$ kcal/mol). In an independent experiment, the average binding stoichiometry was 3.3 ATPs per hexamer ($\Delta H = -4.1$ kcal/mol). The best-fit K_D 's in both ITC experiments were below 100 nM, but the non-linear least-squares errors were greater than the K_D values themselves, indicating that affinity was not well determined.

The binding of fewer than six nucleotides to each HslU homohexamer at saturation could be explained in two ways: (i) once three or four ATPs bind, conformational changes in the two unoccupied subunits preclude further binding; or (ii) roughly two thirds of HslU hexamers bind six ATPs, whereas the remaining HslU molecules fail to bind any nucleotide because of conformational or chemical heterogeneity. In fact, ion-exchange chromatography performed in buffer containing 100 μ M ATP revealed two peaks of HslU E257Q (Fig. 2B), showing heterogeneity of some type. However, in assays that require ATP binding, HslU E257Q from both peaks activated peptide cleavage by HslV and bound a substrate-mimic peptide (Fig. 2C). Moreover, comparison of the absorbance spectra of column fractions containing ATP but no protein with fractions containing HslU

E257Q revealed that both peaks contained about 3.8 ATPs per E257Q hexamer. When either peak-1 or peak-2 protein was rerun on the ion-exchange column, a mixture of the same two peaks was observed, establishing that both forms interconvert. Thus, HslU E257Q can exist in discrete states with different chromatographic properties, but both states bind ATP and are functional in ATP-dependent HslU activities. Because we find no evidence for a form of HslU E257Q that cannot bind ATP, we conclude that ATP binding displays a type of negative cooperativity in which three or four subunits of the hexamer can bind ATP, whereas the remaining subunits do not.

Nucleotide affinity

Nitrocellulose-filter binding was initially used to assay the affinity of E257Q HslU for ^{32}P - γ -ATP (Fig. 3). In one experiment, protein was titrated against a fixed concentration of ATP (Fig. 3A). In another, ATP was titrated against a fixed concentration of E257Q HslU (Fig. 3B). No positive or negative cooperativity was evident in the binding observed in these experiments. Indeed, both sets of data were fit well by a model in which four subunits in the E257Q HslU hexamer each bind ATP with an equilibrium dissociation constant near 0.25 μM . The corresponding equilibrium constant for ATP association gives a binding free energy of approximately -9 kcal/mole. Using ΔH for ATP binding determined in the ITC experiment, we calculate that $T\Delta S$ for ATP binding is about 5 kcal/mol and thus the entropy of ATP association is favorable. Displaced solvent molecules or conformational rearrangements in HslU presumably account for this favorable binding entropy.

We also assayed binding of wild-type HslU or the E257Q mutant to N-methylanthraniloyl (mant) derivatives of ATP, ATP γ S, and ADP by monitoring changes in the fluorescence center-of-mass of these nucleotides (Fig. 4A). E257Q HslU bound mant-ATP and mant-ATP γ S with roughly similar affinities and bound mant-ADP about six-fold more tightly (Table 1). Competition of unmodified ATP, ATP γ S, and ADP for mant-ATP binding to the E257Q protein showed that ADP competed more efficiently than ATP or ATP γ S (Supplemental Figure 7), as expected from direct binding of the mant nucleotides. Wild-type HslU also bound mant-ATP and mant-ATP γ S similarly, albeit less tightly than the E257Q variant, and bound mant-ADP about three-fold more strongly (Table 1). All of the Fig. 4A binding curves were fit well by a simple hyperbolic function, as expected if nucleotide binding to the accessible sites in wild-type or E257Q HslU is not positively or negatively cooperative.

Wild-type HslU bound to mant-ATP and mant-ATP γ S with similar affinities but bound mant-ADP more tightly, suggesting that mant-ATP is not hydrolyzed to produce mant-ADP at a significant rate during the binding experiment. We were initially surprised by this result, as mant-ATP supported HslUV degradation of protein substrates (data not shown). As shown below, however, HslU hydrolyzes ATP very slowly at the low nucleotide concentrations (0.1 μ M) used in these binding assays and we assume that the same is true for hydrolysis of mant-ATP.

E257Q HslU bound mant-ATP, mant-ATP γ S, and mant-ADP more tightly than the wild-type enzyme (Fig. 4A; Table 1). Stronger binding by the mutant is probably a

consequence of replacing the negatively charged glutamate in the nucleotide-binding site with uncharged glutamine, thereby reducing electrostatic repulsion with the β and γ phosphates. This result is consistent with our finding that the mutant but not the wild-type protein purifies with bound nucleotide. Moreover, comparing the affinities of E257Q HslU for ATP and mant-ATP indicates that the mant group weakens binding about 6-fold. By applying an approximate 5-fold correction for the destabilizing effect of the mant group, we estimate that ATP and ATP γ S bind accessible sites in wild-type HslU with an affinity of 0.6-0.7 μ M, whereas ADP binds with an affinity of 0.2 μ M (Table 1). This stronger binding of ADP relative to ATP helps explain why ADP is a potent inhibitor of HslU activity (Rohrwild et al, 1996; Seol et al, 1997).

Although a maximum of four ATP molecules binds E257Q HslU, it seemed possible that ADP binding might be allowed to the remaining empty sites. To test this model, we titrated E257Q HslU against 0.1 μ M mant-ADP in the presence of 1 mM ATP. No mant-ADP binding was detected at the highest protein concentration tested (10 μ M E257Q HslU subunits).

Multiple classes of ATP binding sites

To determine the kinetics of nucleotide dissociation, we allowed complexes containing approximately three mant-ATP molecules and one E257Q HslU hexamer to form, added excess unmodified nucleotide to block further mant-ATP binding, and assayed the rate of dissociation of the fluorescent nucleotide. As shown in Fig. 4B, the dissociation kinetics were biphasic using ATP as the competitor. The same result was observed with

competing ADP (data not shown). In each experiment, the fast phase had a time constant of roughly 20 s and the slow phase had a time constant of about 150 s. These results suggest that there is a minimum of two classes of ATP-bound HslU subunits, even though these subunits bind ATP with similar affinities. Dissociation remained biphasic in the presence of HslV (fast phase 22 s; slow phase 107 s) or HslV and Arc substrate (fast phase 12 s; slow phase 88 s) (Supplemental Figure 8).

Overall, these results support the existence of three classes of HslU subunits. One class fails to bind ATP. Of the two types that bind ATP, one class of subunits releases ATP more slowly than the other. Because both ATP-binding classes have similar ATP affinities, the binding sites that release ATP slowly must also bind ATP slowly and *vice versa*.

ATP hydrolysis

To probe nucleotide interactions with HslU under conditions where ATP hydrolysis provides an additional route for nucleotide escape, we measured changes in the steady-state rate of hydrolysis as a function of ATP concentration (Fig. 4C). Half-maximal hydrolysis activity was observed at a free ATP concentration of roughly 10 μ M. Because this value is about 10-fold higher than ATP concentration needed for half-maximal binding in the absence of hydrolysis, most bound ATP molecules must leave HslU via hydrolysis and subsequent release of ADP/P_i rather than by dissociation of ATP (see Burton et al, 2003). Importantly, a good fit of the concentration dependence of ATP hydrolysis was only achieved by using a Hill constant (n) of 2 in the equation

$\text{rate} = V_{\max} / (1 + (K_M / [\text{ATP}])^n)$. Because we did not observe positive cooperativity in ATP binding to HslU, this result indicates that more than one ATP must bind to an HslU hexamer before the rate of hydrolysis becomes appreciable. Thus, robust ATPase activity requires the binding of multiple ATPs to an HslU hexamer.

Linkage between ATP and peptide binding

We used binding of HslU and HslU E257Q to the substrate-mimic peptide gt1 (MRYFFKKKLRFY) to probe the relationship between recognition and ATP binding (Burton et al, 2005). In fluorescence-anisotropy assays performed in the presence of saturating ATP or ATP γ S, HslU E257Q bound fluorescein-labeled gt1 peptide about 5-fold more weakly than did wild-type HslU (Fig. 5A). The presence of HslV in addition to HslU or the mutant in these binding reactions strengthened peptide binding modestly (Fig. 5A). The finding that the E257Q mutation reduces affinity for gt1 suggests that the Glu²⁵⁷ side chain in the Walker-B motif of wild-type HslU plays a role in linking the energy of ATP binding to the conformational changes that allow binding of this substrate-mimic peptide.

Next, we used a fixed concentration of E257Q HslU and assayed the dependence of gt1-peptide binding on ATP γ S concentration (Fig. 5B; top panel). Although ATP γ S binding was required for peptide binding, the total concentrations of ATP γ S and HslU subunits were too close to calculate free concentrations in a model-independent fashion, and thus to determine if peptide and nucleotide binding were cooperatively linked. To obviate this problem, we repeated this experiment using a weaker-binding ATP derivative, N⁶-

methyl-ATP (Fig. 5B; lower panel). In this experiment, total nucleotide was in excess of HslU sites and thus the free and total nucleotide concentrations were similar. The resulting binding curve showed clear cooperativity with a Hill constant of 1.8, supporting the idea that the conformational changes needed to support substrate recognition require binding of at least two ATP molecules to HslU. When binding was assayed in the presence of HslV₁₂, the concentration of nucleotide required to support binding to the gt1 peptide was reduced more than 10-fold (Fig. 5B). Thus, the HslU•HslV complex must bind nucleotide more tightly than free HslU binds nucleotide.

HslU binds HslV more weakly during ATP hydrolysis

HslU-HslV binding can be assayed by increases in the cleavage of a Z-Gly-Gly-Leu-AMC peptide by HslV (Yoo et al, 1996). When we titrated HslU₆ against 15 nM HslV₁₂ in the presence of saturating ATP, the resulting increase in peptidase activity was fit well by a binding model with an apparent K_d of approximately 75 nM (Fig. 6A, upper panel). In the presence of saturating ATP γ S, binding was substantially stronger ($K_d \leq 15$ nM; Fig. 6A, upper panel). This result could mean that HslV is bound more strongly by HslU•ATP γ S than by HslU•ATP. Alternatively, HslU may bind HslV less tightly when its subunits cycle through the different states of ATP hydrolysis, because a significant period of time is spent in lower-affinity states in which some ATP has been hydrolyzed. However, ATP and ATP γ S supported HslV binding by the hydrolysis-defective E257Q HslU₆ mutant equally well (Fig. 6A, lower panel), supporting the idea that HslU states with lower HslV affinity are populated during the ATPase cycle (see Discussion).

Using surface-plasmon resonance (SPR) to measure HslU•HslV affinity in the presence of ATP γ S, Azim et al (2006) reported a K_D of approximately 1 μ M, a value far weaker than we observe. It is possible that interactions of HslU and/ HslV with the SPR chip surface resulted in an underestimate of affinity. Alternatively, buffer differences may explain the weak binding observed in the SPR experiments, which used Ca⁺⁺ rather than Mg⁺⁺. Indeed, Ca⁺⁺•ATP supported HslU activation of HslV peptidase activity against Z-GGL-AMC only about 1% as well as Mg⁺⁺•ATP (Huang and Goldberg, 1997).

We also titrated HslV against HslU and assayed binding by changes in the rate of ATP hydrolysis. These experiments were performed at 37 °C, because ATP hydrolysis was too slow to obtain reliable data at 25 °C, the temperature of other experiments. HslU•HslV affinity was about 140 nM when just HslU and HslV were present but became 10-fold stronger when an excess of protein substrate (Arc-IA37-st11-titin-ssrA; Burton et al, 2005) was added (Fig. 6B).

HslU₆•ATP activates HslV

To probe the role of nucleotide in HslU activation of HslV, we titrated increasing HslU against 100 nM HslV₁₂ in the presence of 100, 200, 400, and 800 nM ATP γ S and measured Z-GGL-AMC cleavage (Fig. 6C). In this assay configuration, two HslU₆ hexamers can bind each HslV₁₂ dodecamer, and thus saturation should require binding of 200 nM HslU₆, as Kwon et al (2003) have shown that binding of an HslU hexamer activates only the *cis* and not the *trans* HslV ring. Notably, similar total concentrations of HslU₆ resulted in half-maximal activation at each nucleotide concentration, indicating

that binding of ATP γ S, HslU₆, and HslV₁₂ are nearly stoichiometric under these conditions. Starting with 100 nM ATP γ S, 100 nM HslV₁₂, and 1 μ M HslU₆, we found that an additional 8-fold increase in HslV₁₂ did not increase peptidase activity (Fig. 6D). Because nucleotide and HslV binding are linked (e.g., $2U_6 + 2nA + V_{12} \rightleftharpoons 2U_6A_n + V_{12} \rightleftharpoons U_6A_n \cdot V_{12} + U_6A_n \rightleftharpoons U_6A_n \cdot V_{12} \cdot U_6A_n$), this result establishes that almost no free A or U₆A_n is present under the starting conditions. Moreover, because we observe no cooperativity in ATP binding to HslU, the dominant nucleotide-bound species in the 100 nM ATP γ S curve in Fig. 6C should be U₆A \cdot V₁₂ (or U₆A \cdot V₁₂ \cdot U₆A, if positive cooperativity makes binding of the second HslU hexamer substantially more favorable). Thus, binding of a single ATP γ S to an HslU hexamer appears to support HslV binding/activation. If this model is correct, then activation should show a hyperbolic dependence on nucleotide concentration. Indeed, E257Q HslU activation of HslV peptidase activity increased hyperbolically with N⁶-methyl-ATP concentration (Fig. 6E).

By the arguments of the previous paragraph, 100 nM ATP γ S should support HslU₆ binding to half of the HslV rings in 100 nM HslV₁₂, with nucleotide being the limiting factor. Consistently, increasing the ATP γ S concentration increased the observed peptidase activity (Fig. 6C). By our model, however, most if not all of the HslV₁₂ rings should have two HslU hexamers bound in the 200 nM ATP γ S experiment, and yet peptidase activity continued to increase when 400 and 800 nM ATP γ S were used. There are two potential explanations. First, binding of more than one ATP γ S to an HslU hexamer might increase the peptidase activity of the HslV ring to which it binds (see Discussion). Second, binding of the second HslU hexamer to HslV₁₂ might be negatively

cooperative, but the doubly bound species could have more than twice the peptidase activity of the singly bound species.

Discussion

Our results demonstrate that an HslU hexamer binds with substantial affinity to no more than four ATPs. Thus, two potential binding sites in the hexamer appear to be empty, even when nucleotide is saturating. Binding of ATP to the available nucleotide sites occurs non-cooperatively with an equilibrium constant of roughly 1 μ M. However, two phases are observed in dissociation experiments, suggesting the existence of at least two classes of ATP-binding sites. These results suggest that HslU operates by an asymmetric mechanism, with different subunits in the hexamer serving different functional roles. Based on results with other AAA+ enzymes, it is likely that ATP hydrolysis occurs in one or two HslU subunits at a time, with the roles of individual subunits changing in a sequential fashion as repeated cycles of ATP binding, hydrolysis, and ADP/Pi release take place (Martin et al, 2005; Erzberger and Berger, 2006). An important implication of this model is that an all-ADP state or a fully nucleotide-free state of HslU is not part of the normal ATP cycle. Rather, the structural changes in HslU that power translocation and protein unfolding must occur as the enzyme cycles through a saturated ATP state (3 or 4 bound ATPs), a mixed ATP/ADP state, and a partially saturated ATP state.

Our results highlight two crystal structures of the HslU hexamer that show partial occupancy of nucleotide-binding sites (Bochtler et al, 2000). In the 1do0 dimer-of-trimers structure, adjacent subunits in each trimer are nucleotide-free, ATP-bound, and

ATP•Mg⁺⁺-bound, respectively. This structure contains three distinct types of subunits and is thus most compatible with our results. Why is the ATP in this structure not hydrolyzed? One possibility, raised by Wang and colleagues (2001b), is that the four ATPs were misidentified in the crystallographic analysis and are actually ADPs. If just the Mg⁺⁺-free nucleotides were ADP, then this structure might also be inactive because more than two bound ATPs are needed to support hydrolysis. Our results show that ATP hydrolysis requires at least two HslU-bound ATPs, but this is a minimum estimate. Another HslU structure (1do2) is trimer-of-dimers hexamer, with alternating nucleotide-free and AMPPNP-bound subunits. HslU does not hydrolyze AMPPNP, and Mg⁺⁺, which is required for ATP hydrolysis by HslU (Huang and Goldberg, 1997), is also absent in this structure.

In apparent contrast to our results, six nucleotides are bound to the HslU hexamer in 11 crystal structures at resolutions of 3.5 Å or better, (Bochtler et al, 2000; Song et al, 2000; Sousa et al, 2000; 2002; Wang et al, 2001a; Kwon et al, 2003). The bound nucleotide is ADP or dADP in nine of these structures, and is ATP in two structures. How can we reconcile our finding that HslU binds a maximum of four ATPs in solution with structures showing six bound nucleotides? One possibility is that six ATP or ADP molecules can bind HslU if Mg⁺⁺ is absent, as most structures with six bound nucleotides do not have Mg⁺⁺ in the nucleotide-binding site. It is well established that Mg⁺⁺ is required for ATP hydrolysis by HslU, as well as substrate binding, HslV activation, and protein unfolding and degradation (Rohrwild et al, 1996; Huang and Goldberg, 1997; Burton et al, 2005). One HslUV structure (1ofh) does contain six ADPs and Mg⁺⁺, but

there are two rather than the expected single Mg^{++} in each nucleotide site (Kwon et al, 2003). Another possibility is that conformational changes required for HslU to bind six nucleotides occur slowly during the long periods required for crystallization but are not accessible on the much shorter time scales used for solution experiments. In either case, based on our studies, it is unlikely that HslU structures with six bound nucleotides represent functional species in the normal ATPase cycle. Although six nucleotides can obviously bind HslU under some circumstances, this enzyme binds HslV and protein substrates, hydrolyzes ATP, and supports active proteolysis at ATP concentrations where no more than four sites in the hexamer are occupied.

Huang and Goldberg (1997) found that low micromolar ATP concentrations activated peptide cleavage by HslUV, whereas 100-fold higher ATP concentrations were required for degradation of casein, and proposed that that ATP binding to a high-affinity site in HslU reduced affinity for other sites. We find no evidence for this model. Indeed, the three or four HslU subunits that bind nucleotide do so with similar affinities. Rather, we propose that binding of one ATP molecule to HslU supports a conformational change that allows HslV peptidase activation but not robust ATP hydrolysis or the binding of protein substrates. The binding of additional ATPs to the HslU hexamer then drives a second set of conformational changes that activate hydrolysis and substrate binding. When ATP is hydrolyzed, however, apparent “affinity” becomes weaker because dissociation of ADP/Pi rather than ATP becomes the main route of nucleotide release. Our model also explains the results of Yoo et al (1997), who concluded that HslU affinity for ATP γ S was roughly 20-fold stronger than for ATP. By contrast, we find that the actual affinity of

HslU for these nucleotides is almost identical. Again, ATP appears to bind HslU more weakly than ATP γ S because bound ATP dissociates rapidly from the enzyme via hydrolysis, whereas bound ATP γ S does not.

Several of our results support a model in which HslU conformational changes required for HslV activation can occur independently from the structural changes needed for other activities. First, ATP hydrolysis and substrate-mimic binding by HslU require binding of two or more ATPs, whereas HslV activation is supported by a single bound ATP. Second, compared to wild type, the E257Q mutation reduces HslU affinity for the gt1 substrate-mimic peptide but does not reduce affinity for HslV. Thus, different structural changes appear to be involved in these distinct activities.

ATP or ATP γ S binding to HslU is generally required to activate HslV peptidase activity (Rohrwild et al, 1996; Yoo et al, 1996; 1997). Nevertheless, an isolated C-terminal peptide of HslU can activate HslV in the absence of nucleotide (Ramachandran et al, 2002; Seong et al, 2002). In some HslUV crystal structures, this C-terminal peptide assumes an extended conformation that packs between HslV subunits, thereby stabilizing the active conformation of the catalytic site for peptide-bond cleavage (Sousa et al, 2000; 2002; Kwon et al, 2003). In other HslU or HslUV structures, this peptide folds back against the rest of HslU and is not positioned to interact with HslV (Bochtler et al, 2000; Song et al, 2000; Wang et al, 2001a). Because HslU•ADP•HslV complexes are observed in crystal structures (Wang et al, 2001a; Kwon et al, 2003), ATP is clearly not required for HslU binding to HslV. However, crosslinking studies show that HslU•ATP•HslV

complexes are more stable than HslU•ADP•HslV complexes (Yoo et al, 1997). We find that the affinity of HslU for ATP increases when HslV is present. By thermodynamic linkage, ATP-binding must also stabilize HslU•HslV binding. This finding is expected if ATP-dependent conformational changes in HslU release its C-terminal peptides to interact with HslV, thereby stabilizing and activating the proteolytic complex (Sousa et al, 2000; Wang et al, 2001b). An apparent challenge to this model is the finding that the C-terminal tails of an HslU variant lacking the intermediate domain interact with HslV in an ADP-bound form (Kwon et al, 2003). However, each nucleotide site in this structure (1ofh) contains both ADP and P_i , which may mimic ATP.

Our results show that affinity for HslV is about 5-fold stronger when ATP/ATP γ S is bound to HslU but is not hydrolyzed. As discussed above, HslU must cycle through states with three or four bound ATPs, with a mixture of ATP/ADP, and with sub-saturating ATP during hydrolysis. Because affinity for HslV decreases when ATP is hydrolyzed, HslU appears to spend the majority of cycle time in one of the latter states. Indeed, because ADP is a potent inhibitor of HslU-catalyzed ATP hydrolysis (Seol et al, 1997) and binds HslU more tightly than ATP, it would be surprising if ADP release were not the rate-limiting step in ATP hydrolysis. By this model, the mixed ATP/ADP state would be most highly populated because it precedes the rate-determining step. If the C-terminal tails or other regions of ADP-bound HslU subunits in hexamers containing ATP/ADP mixtures did not interact with HslV, then this would provide a straightforward structural basis for the decrease in HslV affinity during ATP turnover. In the related ClpXP system, contacts between the ATPase and peptidase also appear to change dynamically

throughout the ATPase cycle (Martin et al, 2007). Moreover, if functional HslU hexamers are asymmetric, then interactions with HslV are also likely to be asymmetric. The number or strength of molecular contacts with HslV could also vary depending on the number of ATPs bound to an HslU hexamer, explaining the graded activation of HslUV peptidase activity that we observe.

Several aspects of this system favor assembly of productive HslUV•substrate complexes over assembly of HslU•substrate complexes and minimize wasteful ATP hydrolysis. First, binding of HslU to HslV occurs with nanomolar affinity and requires only a single bound ATP. By contrast, the binding of protein substrates to HslU or HslUV requires several bound ATPs and typically occurs with micromolar affinity. Thus, if ATP were scarce, intact protease complexes would assemble, but neither these complexes nor free HslU would bind substrate. Moreover, because several bound ATPs are also required to activate ATP hydrolysis, little energy would be wasted via non-productive hydrolysis under these conditions. At higher ATP concentrations, both substrate binding and ATP hydrolysis can occur. Because HslUV complexes bind ATP more tightly than HslU₆ alone, however, the intact HslUV protease would out compete free HslU₆ for binding available ATP and substrate. This feature of the system should diminish the probability of free HslU hexamers binding substrates, unfolding them at a significant cost in terms of ATP hydrolysis, and translocating them back into solution to refold and/or rebind. Thus, a simple thermodynamic hierarchy ensures that HslU binds one ATP, then binds HslV, and then binds additional ATP and protein substrate prior to hydrolysis-driven unfolding and translocation of the substrate into HslV for degradation.

When ATP is plentiful under normal cellular conditions, HslUV complexes would be stable and have, on average, three to four bound ATPs. However, species with fewer ATPs would be populated transiently as a consequence of hydrolysis and ADP/Pi dissociation. Under these conditions, the need for multiple ATPs for hydrolysis but just one ATP for binding HslV provides a fail-safe mechanism, which may be especially important for an enzyme that binds ADP more tightly than ATP. If, for example, just one ATP remained bound to HslUV during substrate degradation, then hydrolysis/degradation would cease and the complex would remain intact but catalytically inert until additional ATPs could bind. Both this mechanism and the fact that ATP-dependent binding of protein substrate increases HslU•HslV affinity would combine to make degradation highly processive, as is observed (Nishii and Takahashi, 2003).

Methods

Buffers and materials

Buffer D contains 20 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 10% (v/v) glycerol. Nickel-wash buffer contains 50 mM NaHPO₄ (pH 8), 300 mM NaCl, and 10 mM imidazole. Nickel-elution buffer contains 50 mM NaHPO₄ (pH 8), 300 mM NaCl, and 250 mM imidazole. PD buffer contains 25 mM HEPES-KOH (pH 7.6), 5 mM KCl, 5 mM MgCl₂, 0.032% (v/v) Igepal CA-630 (NP-40), and 10% (v/v) glycerol; PDN buffer lacks NP-40 but is otherwise identical. Lysis buffer contains 50 mM Tris-Cl (pH 7.5), 300 mM NaCl, 1 mM EDTA, and 10% (v/v) glycerol. Immediately prior to use, charcoal buffer was prepared by adding 4 M NaCl, 50 mM Tris-Cl (pH 7.5) to 10 g of activated

charcoal (Sigma C-2764) to a final volume of 50 mL. The mixture was shaken for 30 min at room temperature, and the liquid phase was decanted for use.

Sodium salts of ATP and ADP were purchased from Sigma (St. Louis, Missouri); concentrations were determined by absorbance at 259 nm using an extinction coefficient of $15400 \text{ M}^{-1}\text{cm}^{-1}$. The lithium salt of ATP γ S was purchased from Roche Diagnostics (Indianapolis, Indiana). Triethylammonium salts of mant-ATP, mant-ATP γ S, and ADP were purchased from Jena Biosciences GmbH (Jena, Germany); concentrations were determined by absorbance at 355 nm using an extinction coefficient of $5,800 \text{ M}^{-1}\text{cm}^{-1}$. N⁶-methyl ATP was purchased from TriLink BioTechnologies (San Diego, CA); concentration was determined by absorbance at 265 nm using an extinction coefficient of $15,567 \text{ M}^{-1}\text{cm}^{-1}$. Z-Gly-Gly-Leu-AMC was purchased from Bachem.

Proteins

Arc substrates were gifts from Eyal Gur (MIT) and Randy Burton (MIT). HslV-H₆ was purified as previously described (Burton et al, 2005). Cells over-expressing H₆-HslU were lysed as described (Burton et al, 2005), substituting nickel-wash buffer and nickel-elution buffer for buffers A and B. Following elution from Ni²⁺-NTA resin (Qiagen), fractions containing protein were identified by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, California), pooled, and loaded onto a Sephacryl-300 HR26/60 gel-filtration column (Amersham) equilibrated with buffer D containing 0.1 M NaCl. Fractions containing HslU were pooled, loaded onto a HR16/10 Q-Sepharose column equilibrated in buffer D containing 0.1 M NaCl, and eluted with a linear gradient between

buffer D containing 0.1 M NaCl and 1M NaCl. Fractions containing HslU were pooled, concentrated, exchanged into buffer D containing 0.25 M NaCl by PD-10 column chromatography (Amersham), and frozen at -80 °C. H₆-HslU E257Q was purified like the wild-type protein with one exception. Following the nickel column, the eluate containing H₆-HslU E257Q was mixed 1:1 with charcoal buffer for 30 min at 4 °C, and then passed through a 0.2 µm Acrodisc syringe filter (PN4612, Pall Life Sciences, Ann Arbor, Michigan) to remove charcoal particles before continuing the purification. Protein concentrations were determined by UV absorbance at 280 nm using extinction coefficients in units of M⁻¹cm⁻¹ as follows: HslU₆, HslU E257Q, and HslU lacking 7 residues from its C-terminus, 140,820; HslV₁₂, 129,720; Arc-IA37-st11-titin-ssrA, 15,220.

Assays

Unless noted, all assays were performed at 25 °C in PDN buffer. Isothermal titration calorimetry was performed and analyzed as described (Hersch et al, 2005) using a Microcal VP-ITC instrument (Amherst, Massachusetts). All components were degassed, and 159 µM ATP was loaded into a 300-µL syringe and injected in 15 µl aliquots at 10 min intervals into a 1.4-ml cell containing E257Q H₆-HslU (7 µM hexamer). Binding of E257Q H₆-HslU to γ -labeled ³²P-ATP (GE Healthcare) was assayed by nitrocellulose-filter binding as described (Hersch et al, 2005). Fluorescence was measured using a PTI QM-2000-4SE spectrofluorimeter (Lawrenceville, New Jersey). Binding of mant nucleotides to HslU was assayed by changes in the fluorescence center-of-mass (excitation 340 nm; emission 400-500 nm). To assay dissociation kinetics, 2 µM E257Q

HslU hexamer was preincubated with 10 μ M mant-ATP. At time zero, non-fluorescent competitor (5 mM ATP or ADP) was added to prevent rebinding of dissociated mant-ATP and the time course of dissociation was monitored by changes in fluorescence (excitation 340 nm; emission 445 nm). In some dissociation experiments, HslV (2 μ M) or Arc-IA37-st11-titin-ssrA (10 μ M) were also present in the preincubation. For equilibrium-competition experiments, 3 μ M E257Q HslU was added to 100 nM mant-ATP and different concentrations of unmodified ATP, ATP γ S, or ADP were added for 5 min before determining the degree of competition by the reduction in fluorescence.

Ion-exchange chromatography on a MonoQ PC 1.6/5 column (GE Healthcare) was conducted using a SMART system (Pharmacia). The column was equilibrated in 50 mM Tris-Cl (pH 7.5), 100 mM NaCl, 5 mM MgCl₂, 10% glycerol, and 100 μ M ATP. 50 μ L of 15 μ M HslU (hexamer) was loaded and eluted using a linear gradient between equilibration buffer and equilibration buffer with a final concentration of 500 mM NaCl.

ATP hydrolysis was assayed using a coupled system (Burton et al, 2001) at 25 °C or 37 °C in PD buffer. Binding of fluorescein-labeled gt1 peptide to HslU (Burton et al, 2005) was assayed by changes in fluorescence anisotropy (excitation: 467 nm; emission: 520 nm) using motorized Glan Thompson polarizers. Data were collected over 120 s and averaged. When titrating protein against fixed nucleotide, anisotropy values were corrected for scattering. Cleavage of Z-Gly-Gly-Leu-AMC by HslV in the presence or absence of HslU was assayed by changes in fluorescence (excitation: 380 nm; emission: 455 nm) as described (Huang and Goldberg, 1997).

Non-linear least-squares fitting of binding and kinetic data was performed using algorithms implements in KaleidaGraph 3.6.2 (Synergy Software).

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Figure Legends

Figure 1

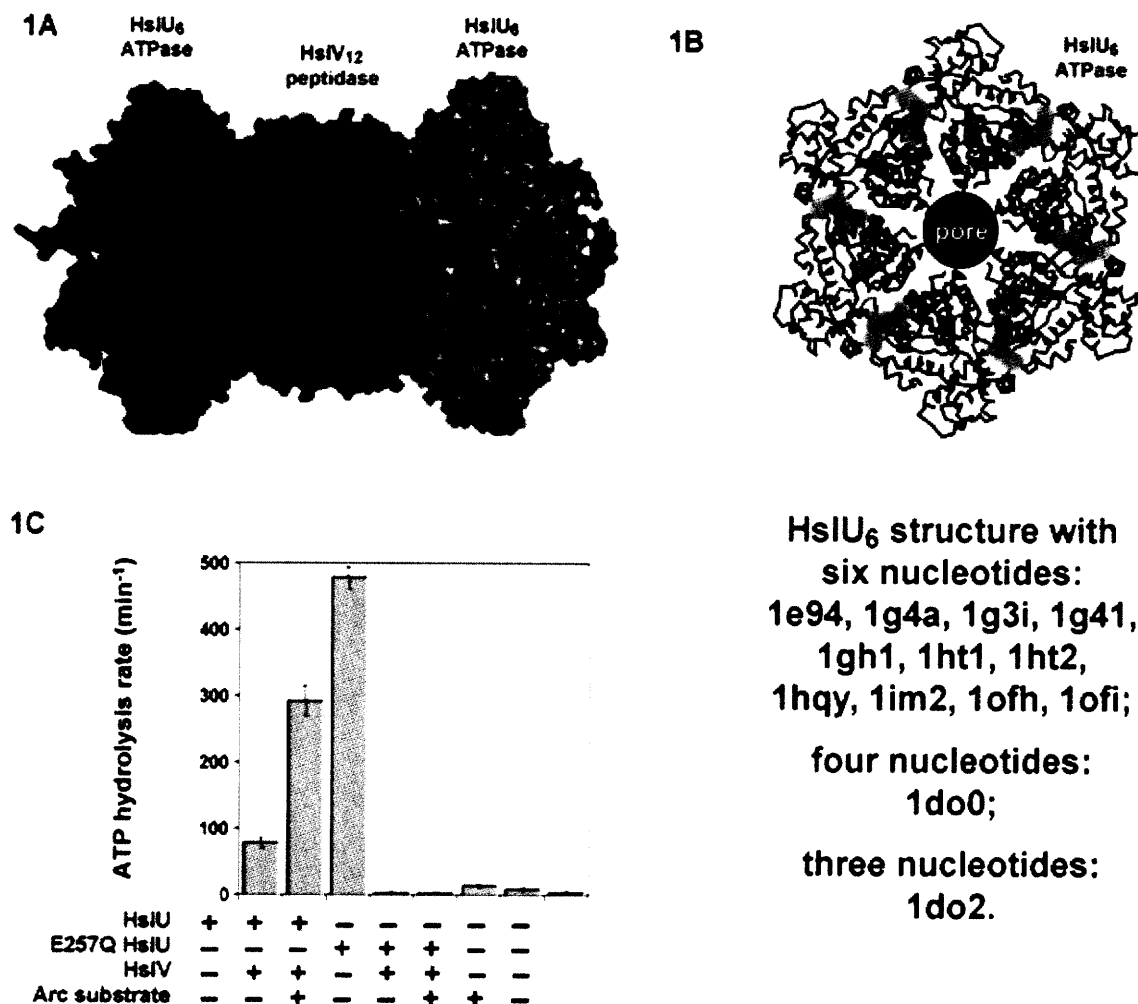


Figure 1. HslU. (A) Two HslU₆ ATPases (blue) can assemble with the HslV₁₂ peptidase (magenta). (B) HslU hexamers have six potential nucleotide binding sites, located at domain and subunit interfaces. From 3-6 nucleotides bind HslU₆ in different crystal structures. (C) Hydrolysis of ATP (2.5 mM) was measured at 37 °C in PD buffer in the presence/absence of HslU or E257Q HslU (0.3 μM hexamer), HslV (0.8 μM dodecamer), and Arc-IA37-st11-titin-ssrA substrate (10 μM). Reactions components were preincubated with 10 μM ATPγS to promote HslU or HslUV association prior to addition

of ATP.

Figure 2

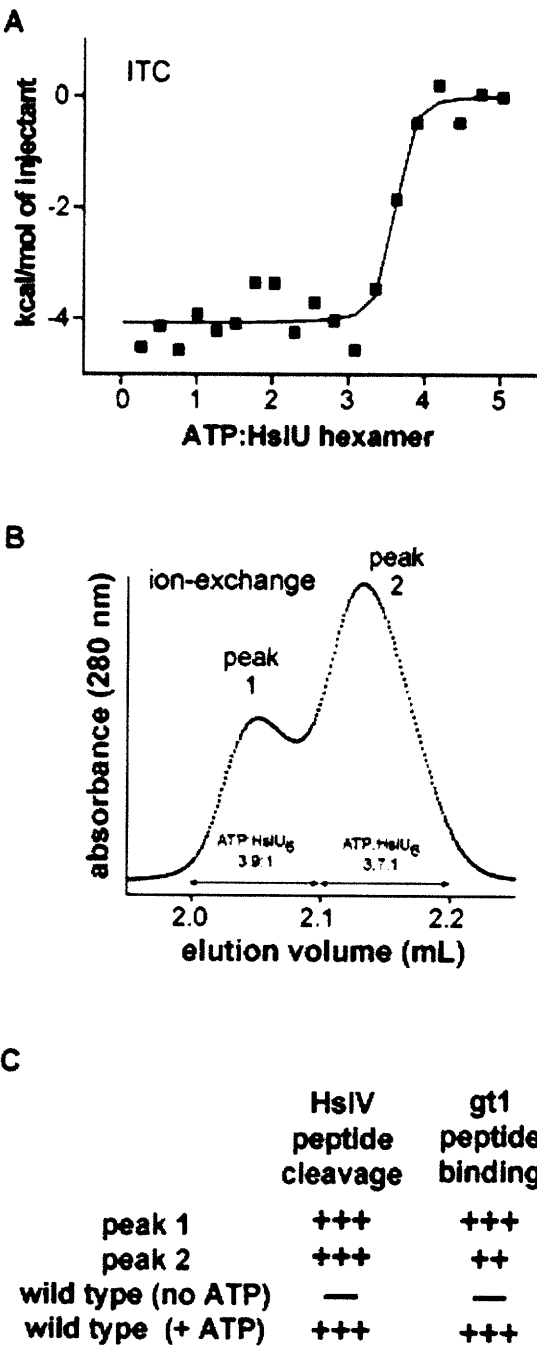


Figure 2. Binding stoichiometry. **(A)** ATP binding to the HslU E257Q mutant was assayed by ITC. 7.5 μ L aliquots of ATP (131 μ M) were injected into 1.5 mL of a solution of E257Q hexamer (7.2 μ M) at 25 °C in NPD buffer. The solid line is a non-linear least squares fit of the data to a single set of sites model ($n = 3.5 \pm 0.05$, $\Delta H = -4.1$ kcal/mol, $K_D = 15 \pm 16$ nM). **(B)** The HslU E257Q mutant (50 μ L of a 12.5 μ M solution of hexamer) was loaded onto a 0.1 mL Sepharose mono-Q column equilibrated in 50 mM Tris•HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl₂, 10% glycerol, and 100 μ M ATP. Equilibration buffer was run for 1 mL and then a linear gradient (2.3 mL total) from equilibration buffer to 50 mM Tris•HCl (pH 7.5), 500 mM NaCl, and 10% glycerol was run. **(C)** Nucleotide dependence of the binding of fluorescein-labeled gt1 peptide (100 nM) to HslU (1 μ M hexamer), HslU E257Q (1 μ M hexamer), or HslU E257Q from the pooled peak-1 or peak-2 samples in panel B. When present, the nucleotide concentration was 1 mM ATP γ S (for HslU) or 1 mM ATP (all other samples).

Figure 3

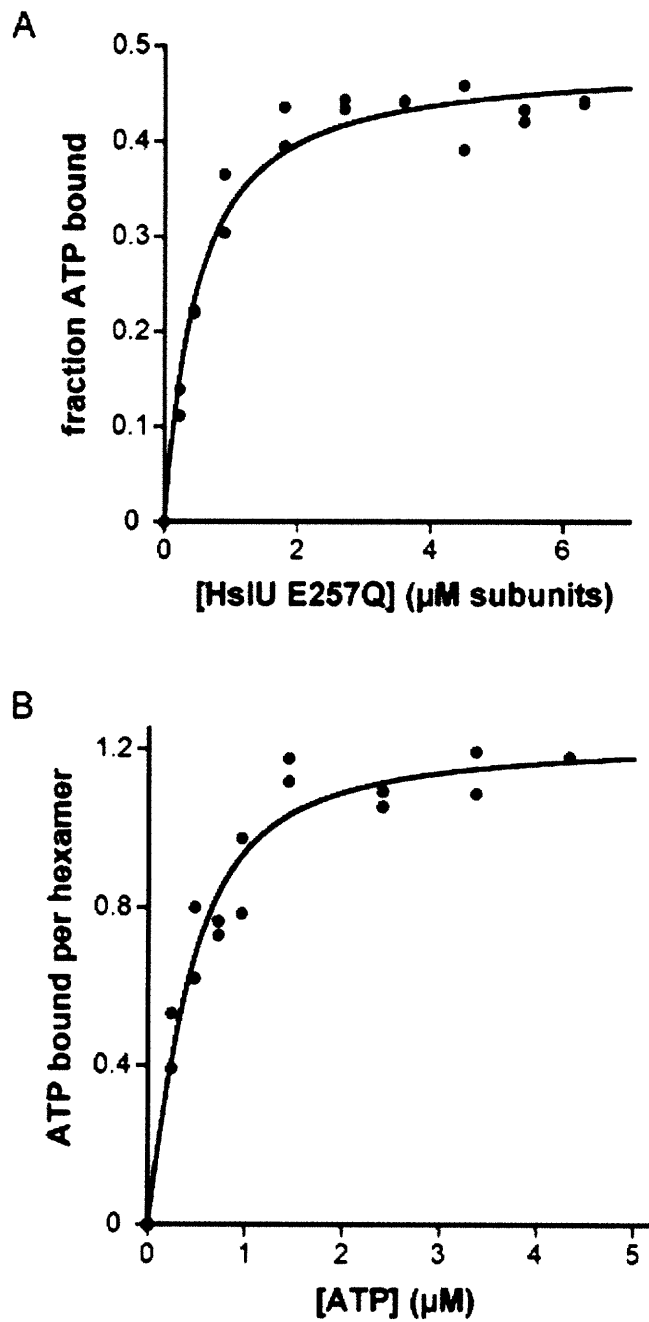


Figure 3. Nitrocellulose filter-binding assays. (A) Binding of increasing E257Q HslU to 0.1 μM ^{32}P - γ -ATP. The solid line is a fit assuming that four of six subunits in the E257Q HslU hexamer bind ATP with an equilibrium dissociation constant of 0.27 ± 0.04 μM per site (B) Binding of increasing ^{32}P - γ -ATP to 0.1 μM E257Q HslU hexamer. The solid line

is a fit to a model assuming four identical ATP binding sites per HslU hexamer with an equilibrium dissociation constant of $0.21 \pm 0.05 \mu\text{M}$ per site. Binding assays were performed at room temperature in PD buffer. Some HslU•nucleotide complexes dissociated during the separation of bound and free ATP in these experiments.

Figure 4

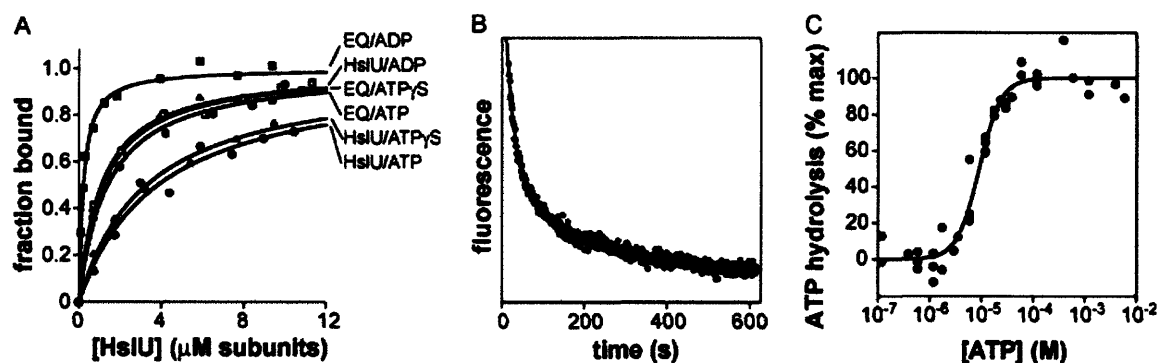


Figure 4. Nucleotide binding, dissociation, and hydrolysis. (A) Equilibrium binding of HslU or E257Q HslU (EQ) to 100 nM mant-ATP (circles), mant-ATP γ S (triangles), or mant-ADP (squares). The fitted lines are for non-cooperative binding. (B) Dissociation kinetics. Following preincubation of E257Q HslU (2 μM hexamer) with mant-ATP (10 μM), excess ATP (5 mM) was added and dissociation was monitored by changes in fluorescence. The fitted line is a double exponential with time constants of 22 ± 1 s (amplitude 70%) and 193 ± 8 s (amplitude 30%) for the two phases. (C) The rate of ATP hydrolysis by HslU (0.3 μM hexamer) was measured as a function of ATP concentration in PD buffer at 25 °C. The line represents the equation: $\text{rate} = 100/(1+([9 \mu\text{M}][\text{ATP}])^2)$.

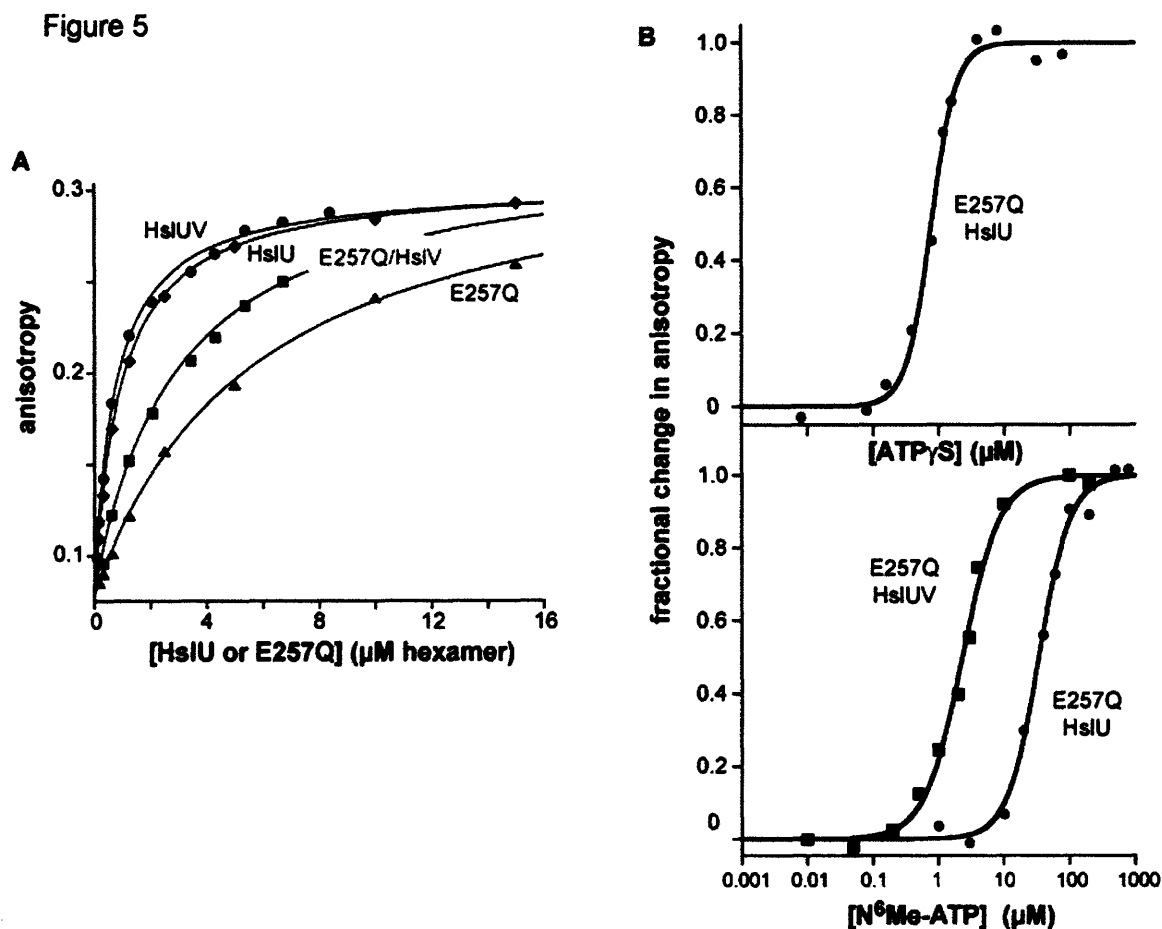


Figure 5. HslU binding to FL-gt1, a fluorescent substrate-mimic peptide. **(A)** Binding of FL-gt1 (100 nM) by wild-type HslU or E257Q HslU in the presence/absence of HslV₁₂. Binding reactions contained 500 μM ATP γ S (HslU) or 500 μM ATP (E257Q HslU) and were performed at 25 °C in PD buffer. The fitted lines are for non-cooperative binding with K_D 's of 0.76 μM (HslUV), 0.93 μM (HslU), 3.0 μM (E257Q HslUV), and 5.6 μM (E257Q). **(B)** Binding of E257Q HslU (1.5 μM hexamer) to FL-gt1 (0.1 μM) as a function of ATP γ S concentration (top panel) or N⁶-methyl-ATP concentration (bottom panel). The data were fitted to the Hill equation. Half-maximal binding occurred at 0.8

μM ATP γS ($n = 2.3$), $34 \mu\text{M}$ N⁶-methyl-ATP without HslV ($n = 1.7$), and $2.3 \mu\text{M}$ N⁶-methyl-ATP with $3 \mu\text{M}$ HslV₁₂ ($n = 1.5$).

Figure 6

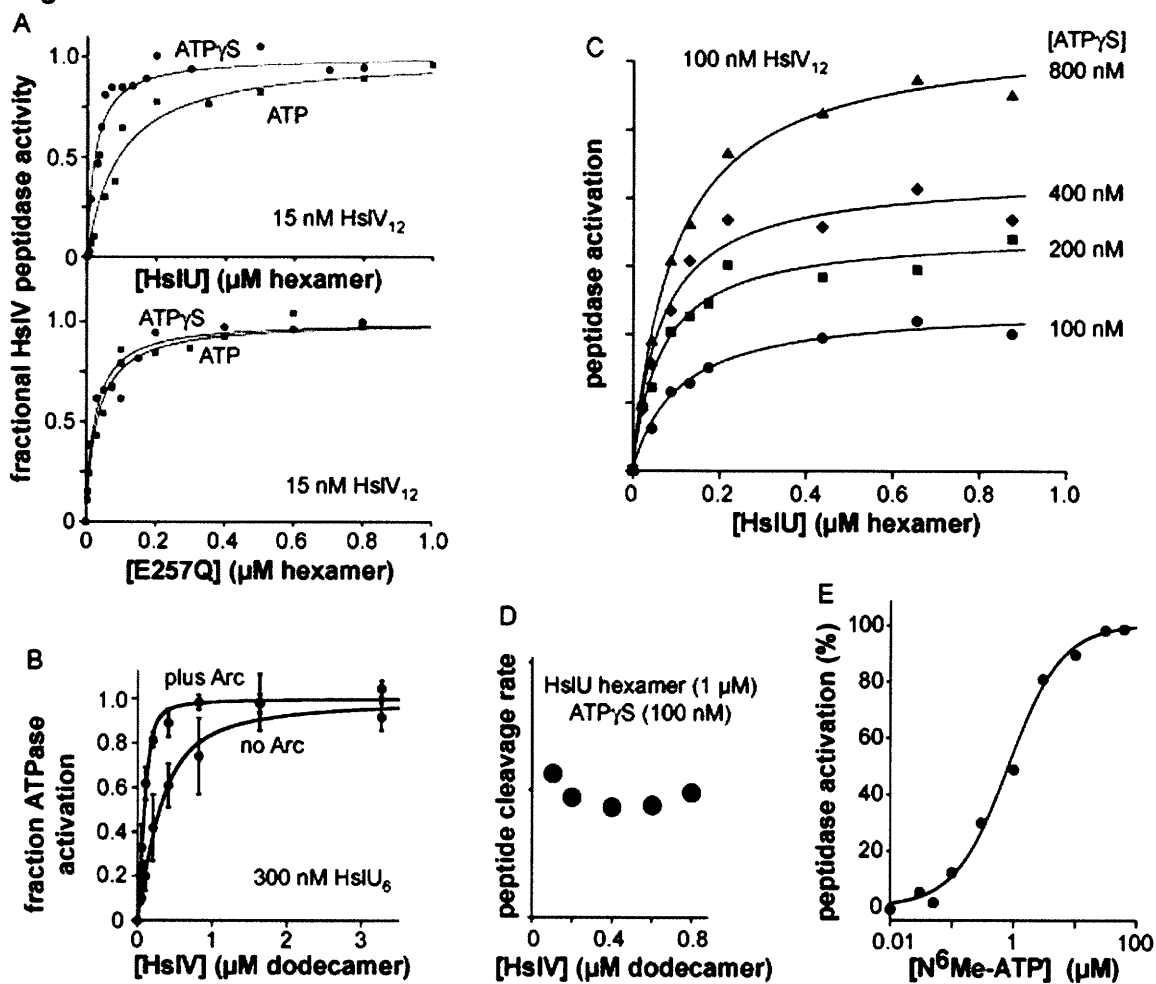


Figure 6. HslU•HslV binding. (A) Binding of HslU (top) or E257Q HslU (bottom) to HslV₁₂ (15 nM) was assayed by changes in the rate of Z-Gly-Gly-AMC peptide cleavage. Reactions contained 1 mM ATP γS or 1 mM ATP and were performed in PD buffer at 25 °C. Data were fit to a quadratic form of a hyperbolic binding isotherm. Apparent K_D values were 12 ± 2 nM (HslU; ATP γS), 78 ± 10 nM (HslU; ATP), 19 ± 5 nM (E257Q

HslU; ATP) and 21 ± 2 nM (E257Q HslU; ATP γ S). **(B)** The presence of a protein substrate (10 μ M Arc-IA37-st11-titin-ssrA) strengthened binding of HslV to HslU (0.3 μ M hexamer), as measured by changes in the rate of HslU ATP hydrolysis. HslUV•Arc complexes were preassembled by incubating for 5 min with 10 μ M ATP γ S at 37 °C. Reactions in PD buffer at 37 °C were initiated by addition of ATP and a regeneration system. **(C)** HslU activation of HslV₁₂ (100 nM) cleavage of Z-Gly-Gly-Leu-AMC (200 μ M) was assayed in the presence of 100, 200, 400 or 800 nM ATP γ S. **(D)** Rates of Z-Gly-Gly-Leu-AMC (200 μ M) cleavage were assayed in the presence of 1 μ M HslU hexamer, 100 nM ATP γ S, and increasing HslV₁₂. **(E)** N⁶-methyl-ATP supports hyperbolic activation of HslU₆ (50 nM) stimulation of HslV₁₂ (200 nM) cleavage of Z-Gly-Gly-Leu-AMC (200 μ M). The solid line is a fit ($R = 0.997$) to the equation activity = $100/(1+([0.85 \mu\text{M}][\text{N}^6\text{-methyl-ATP}]))$.

Table 1. **Nucleotide affinities.**

	ATP (μ M)	ATP γ S (μ M)	ADP (μ M)	mant- ATP (μ M)	mant- ATP γ S (μ M)	mant- ADP (μ M)
HslU	<i>0.65</i>	<i>0.55</i>	<i>0.19</i>	3.8	3.2	1.1
E257Q	0.24	<i>0.21</i>	<i>0.04</i>	1.4	1.2	0.2

Values are equilibrium dissociation constants of nucleotides for a single site in an HslU hexamer. Values for mant nucleotides are taken from the fits of the experiments shown in Fig. 4A. The value for ATP and E257Q HslU is an average of the values determined from the fits of the experiments shown in Fig. 3A and 3B. Values in italics were calculated by dividing the affinities for the corresponding mant-nucleotide by 5.8, the ratio of the affinities of E257Q HslU for mant-ATP versus ATP.

Appendix 2.1.

HslV activation in the absence of nucleotide

HslV activation by HslU₆ requires ATP in most instances and does not occur with ADP (Yoo et al., 1996). However, partial activation has been observed with Mg⁺⁺ but no ATP present (Huang and Goldberg, 1997). I assayed Z-Gly-Gly-Leu-AMC peptide cleavage by 15 nM HslV₁₂ alone or with different combinations of HslU₆ (50 nM), ATPγS (250 μM), and Mg⁺⁺ (12 mM). As expected, the complete mixture gave the strongest activity, but approximately 10% activity was observed when just HslU and Mg⁺⁺ were present; all other combinations gave less than 1% activity. To probe the strength of ATP-independent activation, I titrated HslU against HslV in the absence of nucleotide (Fig. 7), and observed half activation at less than 10 nM HslU₆, indicating a strong interaction. When an HslU variant missing seven C-terminal residues was used in this experiment, no activation of HslV was observed (Fig. 7). Thus, the C-terminal tails of HslU are required for ATP-dependent and ATP-independent HslV activation.

What accounts for the ATP-independent activation of HslV, which occurs at very low HslU concentrations? HslU hexamers dissociate into dimers when nucleotide is absent (Yoo et al., 1996; Yoo et al. 1997; Kessel et al., 1996; Rohrwild et al., 1997). Hence, ATP-independent activation of HslV could be explained if the C-terminal peptides of a dissociated HslU dimer bound and activated HslV. The C-terminal peptide of a single HslU subunit should bind HslV no more tightly than an isolated peptide ($\approx 20 \mu\text{M}$; Ramachandran et al., 2002) but the increased “effective” concentration of the second peptide in an HslU dimer ($C_{\text{eff}} > (2 \cdot 10^{-5} \text{ M})^2 / (10^{-8} \text{ M}) = 40 \text{ mM}$) could account for the

strong binding (< 10 nM) in the absence of nucleotide. Because ATP is always present in living cells, however, nucleotide-independent activation of HslV by HslU is probably physiologically irrelevant.

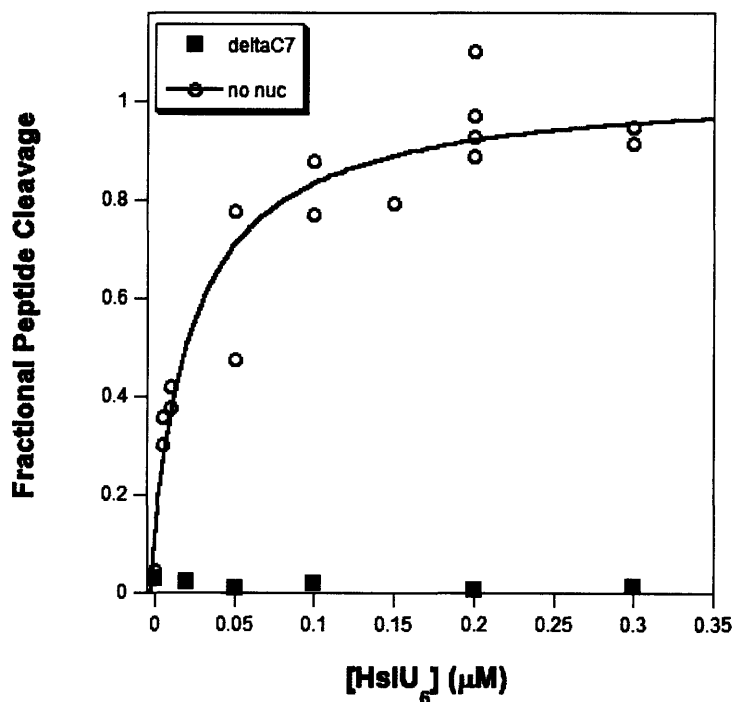
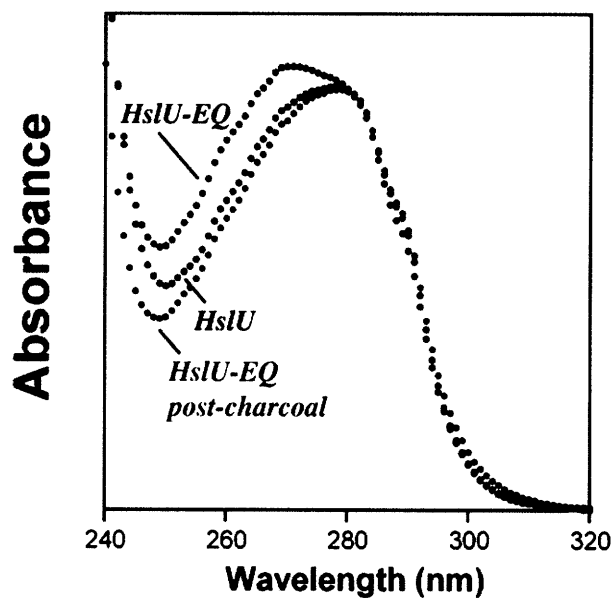
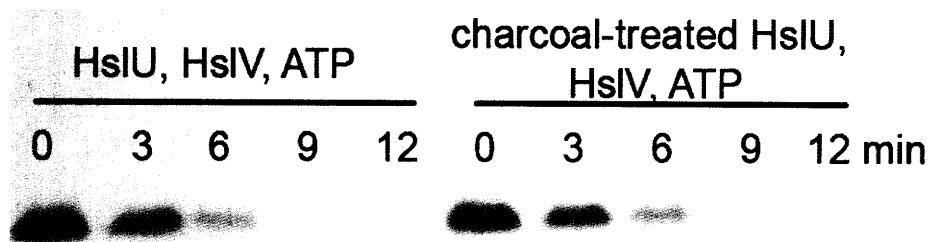


Figure 7. ATP-independent HslU•HslV binding. HslV₁₂ (15 nM) was incubated with Z-Gly-Gly-Leu-AMC (100 μM) and HslU in the absence of nucleotide (circles) or HslU lacking its 7 C-terminal residues (squares).

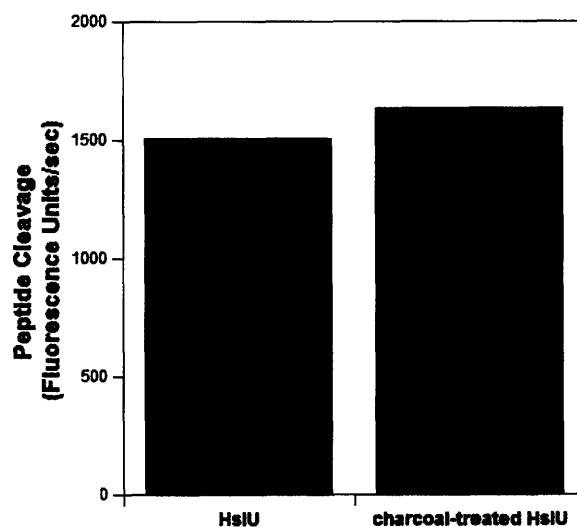
Supplemental Figures



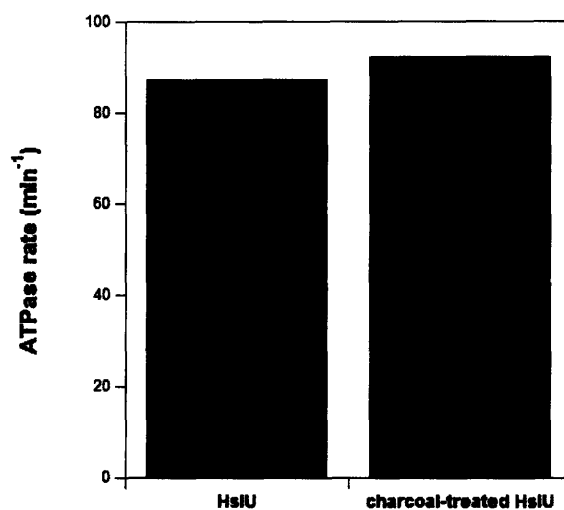
Supplemental Figure 1. Charcoal-treated HslU-EQ has a similar absorbance to wild-type HslU.



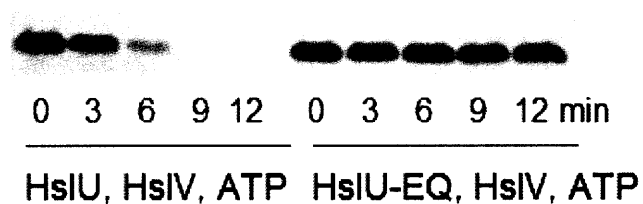
Supplemental Figure 2. Charcoal-treated HslU supports normal HslV mediated degradation of Arc repressor. Numbers represent time of degradation in minutes. Reactions were performed at 37 °C in PD buffer with Arc repressor (10 μ M), HslU (0.3 μ M hexamer), HslV (0.8 μ M dodecamer), and ATP (2.5 mM).



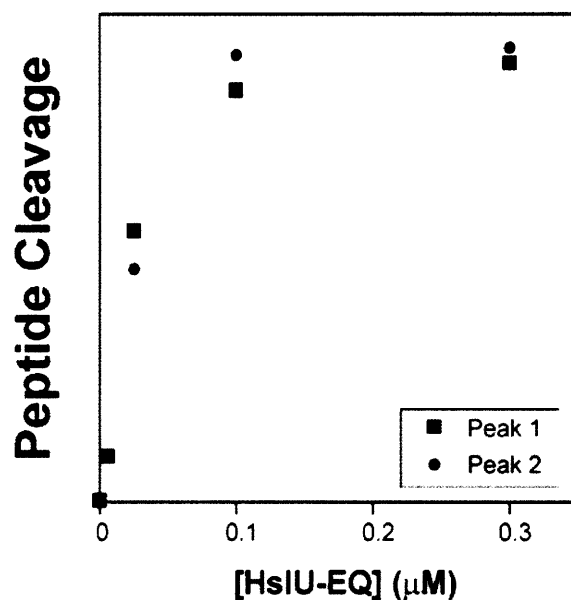
Supplemental Figure 3. HslU and charcoal-treated HslU (50 nM hexamer) both activate HslV₁₂ (15 nM) cleavage of Z-GGL-AMC activity to similar extents in the presence of 2 mM ATP γ S.



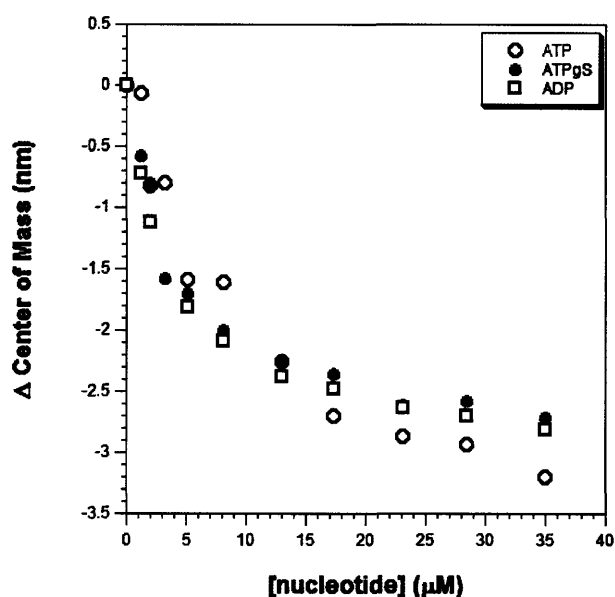
Supplemental Figure 4. HslU and charcoal-treated HslU (0.3 μ M hexamer) both hydrolyze ATP (2.5 mM) at similar rates.



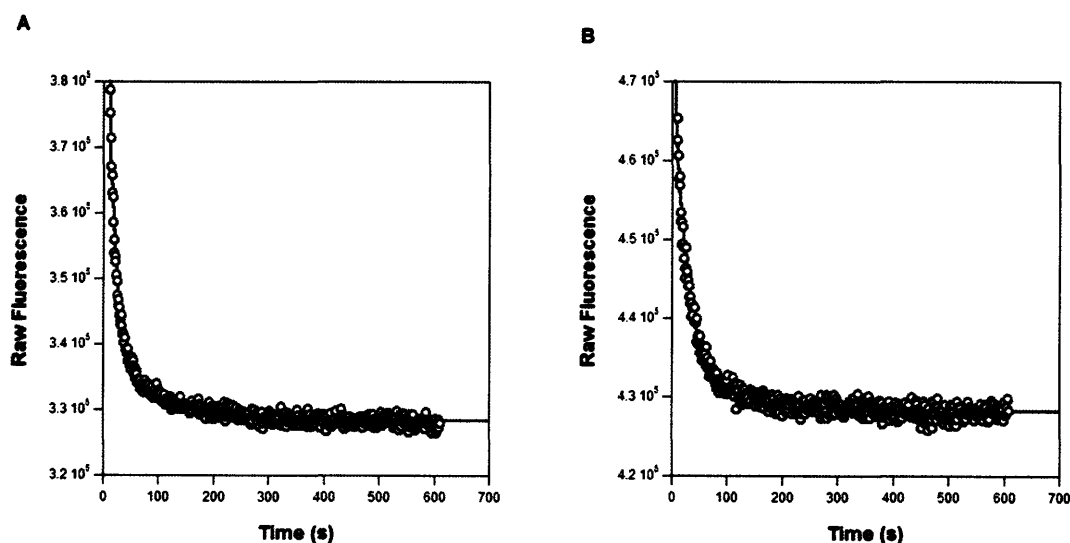
Supplemental Figure 5. HslU E257Q is unable to support HslV degradation of Arc repressor. Reactions at 37 °C in PD buffer contained Arc repressor (10 μ M), HslU (0.3 μ M hexamer), HslV (0.8 μ M dodecamer), and ATP (2.5 mM).



Supplemental Figure 6. HslU E257Q from the peak 1 and peak 2 pools from the ion-exchange chromatography experiment shown in Fig. 2b stimulated HslV (15 nM dodecamer) cleavage of Z-GGL-AMC to comparable extents in the presence of 100 μ M ATP γ S.



Supplemental Figure 7. Competition of ADP, ATP, and ATPγS for binding of 100 nM mant-ATP to HslU E257Q (3 μM monomer equivalents) in PDN buffer at 25 °C was monitored by changes in fluorescence center-of-mass.



Supplemental Figure 8. Dissociation kinetics of mant-ATP (10 μM) from E257Q HslU (2 μM hexamer) in the presence of HslV or HslV/Arc. Dissociation was started by addition of 5 mM ATP and monitored by changes in fluorescence. The resulting data were fit to a double exponential. (A). In the presence of 5 μM HslV₁₂, time constants

were 22 s (amplitude 85%) and 107 s (amplitude 15%). **(B)**. In the presence of 2 μM HslV₁₂ and 10 μM Arc, time constants were 12 s (amplitude 88%) and 88 s (amplitude 12%).

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CHAPTER THREE

HslU nucleotide binding site and pore-2 loop mutants

Chapter 3

Introduction. As I demonstrated in chapter 2, the nucleotide state of HslU affects ATP hydrolysis, substrate interactions, and HslV binding and peptidase activation. To probe in more detail how the nucleotide-binding site affects these activities, I have determined the consequences of mutating two conserved arginines in *E. coli* HslU, the arginine “finger” (R325) and sensor-2 arginine (R393). Both residues have been shown to play key roles in nucleotide binding, sensing, hydrolysis, and subunit oligomerization in other AAA+ or AAA enzymes (Ogura et al., 2004). I have also studied mutations in the pore-2 loop of HslU. In ClpX hexamers, this pore loop plays roles in recognition of protein substrates and in mediating ATP-dependent dynamic communication with the ClpP peptidase (Martin et al., 2007).

The sensor-2 arginine is only conserved in the AAA+ subfamily of ATPases and contacts bound nucleotide in some but not all crystal structures of HslU. Mutation of sensor-2 arginines can affect ATP binding, ATP hydrolysis, and hexamer stability but these effects also vary depending upon the AAA+ enzyme (Ogura et al., 2004). The arginine finger forms part of the nucleotide-binding site created by amino acids from the adjacent subunit. It is positioned to interact with the γ phosphate of the nucleotide triphosphate, similar to arginine-finger residues in GTPase-activation proteins (Rittinger et al., 1997; Scheffzek et al., 1997; Tesmer et al., 1997). In different crystal structures of HslU, the arginine finger (R325) side chain is near but not in contact with bound nucleotide. Some studies of R325 and R393 mutations have been reported (Song et al., 2000). For example,

compared to wild-type HslU, the R325E and R393A mutants were found to have less than 20% activity in ATP hydrolysis and in assays of HslV mediated peptide, casein, and SulA degradation. However, gel-filtration studies suggested that the R325E mutant failed to hexamerize and that the R393A mutant formed hexamers poorly.

The pore-2 loop of HslU (residues 263-271; Fig. 1) forms the bottom portion of the central channel of the hexamer and is close to the axial portal of HslV in complexes. In the related protease ClpXP, the pore-2 loop is important for ClpP binding and communication (Martin et al., 2007). The D201N mutation in the ClpX pore-2 sequence decreased ClpX-ClpP affinity roughly 15-fold, as did replacing the loop with a glycine/serine rich linker (Δ pore-2). These mutations also stimulated basal ATPase activity by ClpX but eliminated ClpP repression of ATP hydrolysis. Both ClpX pore-2 mutants also caused defects in ClpXP degradation of specific substrates. To determine if the pore-2 loop of HslU played a similar role in ATPase-peptidase communication and/or substrate recognition, I constructed pore-2 mutations in HslU and assayed their effects on HslV affinity and substrate interactions.

Walker B										pore 2									
I	D	K	I	C	K	R	G	E	S	S	G	-	-	P	D	V	S	HslU_ecoli	
258	259	260	261	262	263	264	265	266	267	268	269			270	271	272	273		
I	D	K	I	C	K	K	G	E	Y	S	G	-	-	A	D	V	S	HslU_haein	
259	260	261	262	263	264	265	266	267	268	269	270			271	272	273	274		
I	D	K	I	S	R	L	S	E	N	R	S	I	T	R	D	V	S	ClpX_helpy	
216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233		
I	D	K	I	S	R	K	S	D	N	P	S	I	T	R	D	V	S	ClpX_ecoli	
186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203		

Figure 1. Partial sequence alignment between HslU and ClpX. The pore-2 region is highlighted.

Results and Discussion

Arginine finger and sensor-2 mutations.

I created, expressed, and purified HslU variants bearing the R325A, R325K, R393A, and R393K mutations, and assayed the abilities of these mutants to bind mant-ATP γ S and mant-ADP, to hydrolyze ATP in the presence and absence of HslV, to bind HslV (as assayed by changes in ATP hydrolysis), to stimulate HslV hydrolysis of Z-GGL-AMC, and to bind the fluorescent peptide substrate mimic gt1. A summary of these results is shown in Table 1.

	nucleotide binding affinity (μ M)		ATPase	Fold ATPase stim by HslV	Apparent HslUV Kd (μ M)	gt1 Kd	HslV stim %	degrades Arc?
	mant-ADP	mant-ATP γ S						
WT	1.1	3.3	100%	6.6	0.15	0.95	100	yes
R393A	3.7	2.5	9%	2	1.5	4.7	20	—
R393K	3.5	3.1	9%	2	0.1	0.64	100	—
R325A	2.3	8.3	20%	≤ 2	unclear	6.5	20	—
R325K	9	5.2	40%	2	0.3	7	15	—
D271N	1	2.8	50%	6.2	0.3	>9.7	80	no
deltaPore2	0.9	3.4	50%	4.5	0.5	0.62	70	yes

Table 1. Biochemical activities of wild-type and variant HslU enzymes bearing Arg-finger, sensor-2 arginine, and pore-2 mutations.

Binding affinities for mant-ATP γ S and mant-ADP were assayed for the R325A, R325K, R393A, and R393K mutants by changes in fluorescence, as described in chapter 2. A significant reduction in ATP γ S affinity (2-3 fold) was only observed for the R325A and R325K mutations (Table 1). These arginine-finger mutations also decreased affinity for mant-ADP (Table 1), suggesting that the arginine-finger side chain (R325) plays roles in both ATP and ADP binding. Interestingly, the R393A and R393K mutations reduced binding affinity for mant-ADP but not for mant-ATP γ S (Table 1), suggesting a specific

role in sensing the identity of bound nucleotide. Indeed, structural analysis indicates that the sensor-2 arginine side chain can interact with the β phosphate of ADP (Trame et al., 2001). Because a β phosphate is common to ADP and ATP, my results suggest that the sensor-2 arginine interacts more strongly with this group in ADP.

The R393A and R393K mutations reduced HslU ATP hydrolysis rates to 9% of the wild-type level (Table 1). These defects are unlikely to result from changes in ATP binding (as these mutants bound mant-ATP γ S normally) or from decreased affinity for ADP (as judged by mant-ADP binding). Indeed, if ADP release is the rate-limiting step in ATP hydrolysis (see chapter 2), then decreased ADP affinity might be expected to enhance not to depress the hydrolysis rate. Thus, the R393 side chain probably plays a modest catalytic role in ATP hydrolysis. The R325A and R325K mutations reduced ATP-hydrolysis rates to 20% and 40%, respectively, of wild-type HslU (Table 1). Because both of these mutations weaken ATP binding, their detrimental effects on ATP hydrolysis could be caused by changes in the geometry of ATP binding, which make attack of a water molecule on the γ phosphate less favorable.

At saturating concentrations of HslV, the weak ATPase activities of the R325A, R325K, R393A, and R393 mutants were stimulated about 2-fold, whereas ATP hydrolysis by wild-type HslU was stimulated more than 5-fold (Table 1; Fig. 2). Thus, the finger and sensor-2 arginines may play some role in mediating HslU-HslV communication. I attempted to estimate HslV affinities for the arginine finger and sensor-2 mutants from the concentration dependence of ATPase activation (Fig. 2). The R393K mutant bound

HslV slightly more tightly than wild-type HslU (Table 1). This may also be true for the R393A variant, but the ATPase data was very noisy and could not be fit to a binding isotherm. The R325A and R325K mutants appeared to bind HslV more weakly than wild type, but these data were also somewhat erratic and the corresponding fits were probably too poor to draw any confident conclusions (Table 1; Fig. 2).

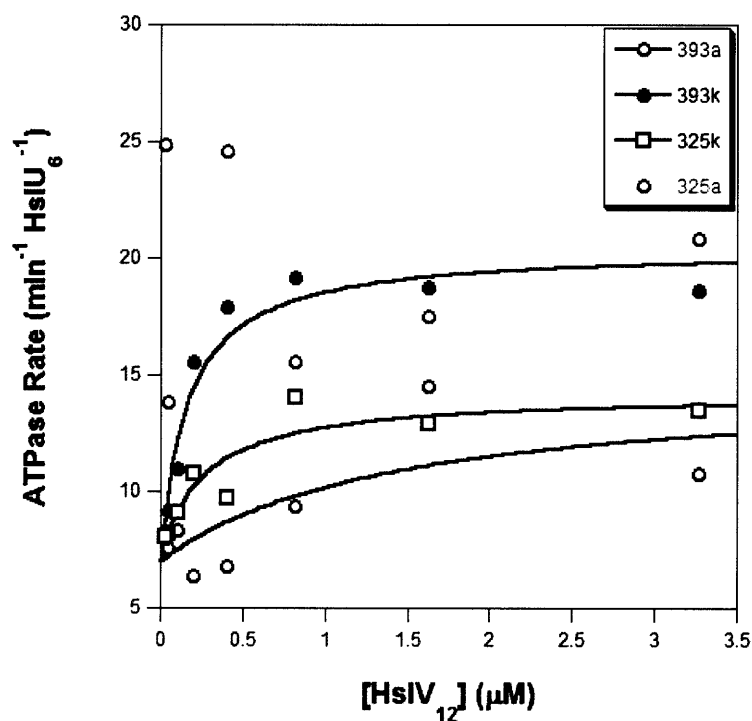


Figure 2. HslV stimulation of ATP hydrolysis by arginine-finger and sensor-2-arginine variants of HslU.

The R325A, R325K, R393A, and R393K mutants all bound the substrate mimic gt1 peptide (Fig. 3; Table 1). However, except for the R393K variant, the gt1 affinities obtained by fitting the titration curves were weaker than wild-type HslU by factors of 5-

to 7-fold. Interestingly, the anisotropy values at binding saturation were much lower for all of the arginine-finger and sensor-2-arginine mutants than for wild-type HslU (Fig. 3). This result suggests that these mutants bind the gt1 peptide somewhat differently than wild type. For example, the mutants might bind gt1 in a manner that allows the fluorescent dye more rotational freedom than in the wild-type complex. Hence, the wild-type arginine finger and sensor-2 arginines probably play roles in coordinating ATP binding with the conformational changes that allow tight and/or proper substrate binding.

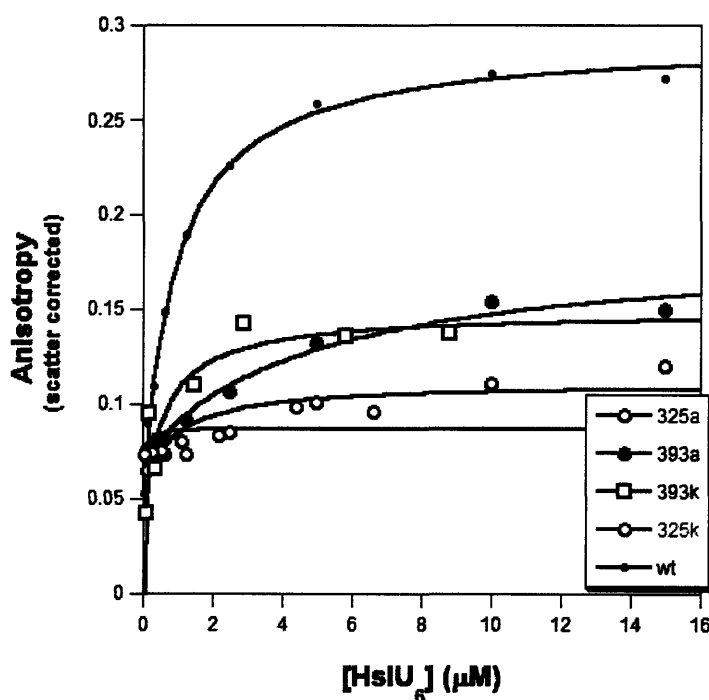


Figure 3. gt1 binding by wild-type and mutant variants of HslU.

In ClpX, the corresponding sensor-2-arginine mutation caused more severe phenotypes than in HslU. For example, the Arg→Lys substitution at the ClpX sensor-2-arginine position reduced ATP hydrolysis rates to less than 1% of the wild-type value, eliminated

binding of ClpX to ClpP, and prevented recognition of the ssrA degradation tag (Joshi et al., 2004). These results might mean that the wild-type sensor-2 arginine plays a more important role in ClpX than it does in HslU. Alternatively, the Arg→Lys mutation may be more disruptive in ClpX for structural reasons. For example, crystal structures of ADP-bound forms of HslU form closed hexameric rings, whereas an ADP-bound form of ClpX forms a distorted lock-washer structure that forms a continuous helical spiral in the crystal lattice (Kim and Kim, 2003). It is possible, therefore, that the sensor-2-arginine mutation in ClpX causes conformational changes, similar to those induced by ADP binding, which result in disruption of all activities that depend upon a closed hexameric conformation.

Pore-2 mutations

To test the role of the pore-2 loop in HslU function, I constructed variants with either the D271N substitution or a pore-2 deletion/substitution (Δ pore-2; HslU residues 263-271 replaced with a GGSSGG sequence). These mutants were tested for nucleotide binding, ATP hydrolysis, HslV and gt1 binding, and degradation of Arc repressor (Table 1; Figures 4-6).

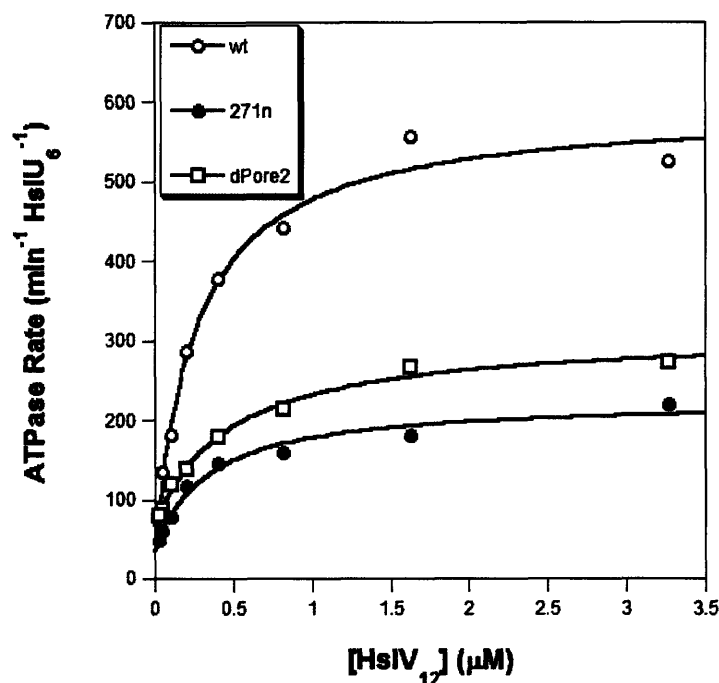


Figure 4. HslV stimulation of ATP hydrolysis by wild-type and pore-2 loop variants of HslU.

Both of the HslU pore-2 mutants bound mant-ATP γ S and mant-ADP with affinities similar to wild-type HslU and each showed maximal ATP-hydrolysis rates approximately 50% lower than the wild-type level (Table 1; Fig. 4). Thus, the pore-2 sequence of HslU plays some role in regulating ATP hydrolysis, as does the corresponding pore-2 sequence in ClpX (Martin et al., 2007). However, the pore-2 mutations in ClpX increased rather than decreased the basal rate of ATP hydrolysis. Moreover, the pore-2 mutations in ClpX reduced affinity for ClpP substantially and prevented ClpP repression of ATPase activity (Martin et al. 2007). In HslU, by contrast, the D271N mutation and the pore-2 deletion/substitution caused only modest decreases in HslV affinity and the ATPase activity of the mutant proteins still responded to HslV binding in a more-or-less wild-type

fashion (Table 1; Fig. 4). Hence, the role of the pore-2 loop in mediating ATPase-peptidase binding and communication clearly seem to be different for HslUV and ClpXP. This result may not be surprising as the 6-fold symmetric HslV and 7-fold symmetric ClpP have unrelated structures and therefore must interact with the homologous HslU and ClpX ATPases in rather different fashions.

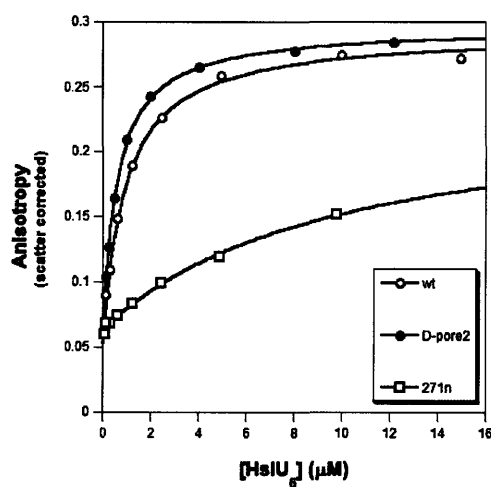


Figure 5. Equilibrium binding of HslU and the pore-2 loop variants to the gt1 substrate-mimic peptide.

The conservative pore-2 mutation D271N reduced HslU affinity for the gt1 peptide more than 10-fold and slowed HslUV degradation of Arc repressor substantially (Fig. 5; Fig. 6; Table 1). By contrast, the HslU Δ pore-2 mutant, in which a GGSSGG linker replaces D271 and other loop residues, bound the gt1 peptide and supported HslV degradation of Arc at near wild-type levels (Fig. 5; Fig. 6).

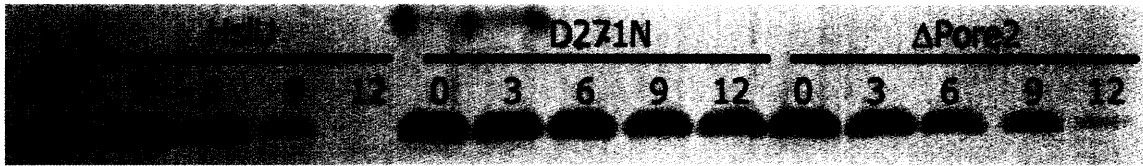


Figure 6. The D271N mutant supports only slow degradation of Arc repressor, whereas the Δ pore-2 mutant displays activity similar to wild-type HslUV as assayed by SDS-PAGE. Numbers represent time of degradation in minutes. Reactions at 37 °C in PD buffer contained Arc repressor (10 μ M), HslU or variants (0.3 μ M hexamer), HslV (0.8 μ M dodecamer), and ATP (2.5 mM).

The observed near loss-of-function caused by the conservative Asp→Asn mutation but retention of function for the more radical mutant in which D271 is absent is clearly surprising. Apparently, removing a section of the pore-2 loop is less deleterious than replacing a single carboxyl group with a carboxamide group. Mary Lee, a graduate student in our lab, recently found that replacing the HslU pore-2 loop with the ClpX loop or a truncated ClpX loop resulted in severe gt1-binding and Arc-degradation phenotypes similar to those I observe for the D271N HslU mutant (personal communication). Transplanting the ClpX loop into HslU left the D271 position unaltered.

The majority of the mutational results suggest an important role for the pore-2 loop of HslU in substrate recognition. Moreover, mutations in the pore-2 loop of ClpX prevented degradation of ssrA-tagged substrates but not other classes of substrates, again supporting an important role for the pore-2 loop in substrate recognition (Martin et al., 2007). However, the properties of a single HslU mutant (the Δ pore-2 variant) suggest that the pore-2 loop sequence can be changed rather dramatically with no apparent effect on gt1 or Arc substrate recognition. At present, therefore, the role of the HslU pore-2 loop in substrate recognition is confusing.

Methods and Materials

Mutants were purified and most assays were conducted as reported in chapter 2. Assays of Arc degradation monitored by SDS-PAGE were performed as described (Burton et al., 2005).

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CHAPTER FOUR

HslUV cysteine mutants and work towards an HslUV FRET assay

Chapter 4

Introduction

In chapter 2, I studied interactions between HslU and HslV by several functional assays, including changes in HslU-ATPase activity upon HslV binding and changes in HslV-peptidase activity upon HslU binding. Although useful, these assays were limited to conditions that support the enzymatic activities that were used as readout of the interaction. As a result, I was not able to study the interaction in the absence of nucleotide or in the presence of ADP.

Historically, several additional assays have been used to study HslU•HslV interactions, including gel filtration, crosslinking with bifunctional reagents, and surface plasmon resonance (Yoo et. al, 1997; Azim et al., 2005). Each technique has problems. For example, neither gel filtration nor crosslinking provides a quantitative analysis of binding affinity. Surface plasmon resonance (SPR) has been used to monitor HslUV binding affinities under a variety of conditions (Azim et al., 2005), and should provide quantitative information. However, the published SPR experiments gave an HslU•HslV binding affinity of roughly 1 μ M, whereas the solution experiments presented in chapter 2 gave an affinity more than 50-fold tighter. This discrepancy may be due to problems of HslU/HslV adsorption to the SPR chip surface or to differences in the buffers used (the SPR experiments substituted calcium for magnesium, which has been shown to be an important cofactor in allowing HslU to adopt the ATP-bound conformation (Burton et al., 2005)).

Because of the problems noted above, I tried to develop a solution-based physical assay based on fluorescence resonance energy transfer (FRET) that could be used to better understand HslU•HslV complex association and dissociation under a variety of experimental conditions. In the end, I was not successful in establishing a FRET assay but I did construct active HslU and HslV mutants that contain single-cysteine substitutions, which may be useful for future studies.

Results and Discussion

To allow site-specific labeling of HslU and HslV with fluorescent dyes, I wanted to eliminate the wild-type cysteines in both proteins and then introduce mutant cysteines at specific positions in each molecule. However, it was initially reported that both wild-type cysteines in HslU (Cys²⁶¹ and Cys²⁸⁷) and the single cysteine in HslV (Cys¹⁵⁹) were required for oligomerization, as treatment of either enzyme with *N*-ethylmaleimide resulted in dissociation into monomers (Yoo et al., 1998). Moreover, when the cysteines in *E. coli* HslU were mutated individually to valine, the C261V mutant retained activity but the C287V mutant could no longer hydrolyze ATP (Yoo et al., 1998). The activity of the C261V mutant was not surprising, as HslU orthologs frequently contain Ala and sometimes Val at the homologous position. The inactivity of the C287V mutant was probably not due to a requirement for cysteine, as many HslU orthologs have Ser or Thr in place of C287. I constructed individual C261A and C287S mutants of *E. coli* HslU and assayed their activities. Both variants chromatographed as hexamers during gel filtration (data not shown) and both mutants retained near wild-type levels of ATP hydrolysis

(Figure 1). I also constructed and purified the HslU double mutant C261A/C287S, which was active in ATP hydrolysis, Arc degradation, and peptidase activation of HslV (data not shown). Thus, as expected from phylogenetic comparisons, the wild-type cysteines in *E. coli* HslU can be replaced by appropriate residues with little if any loss of function.

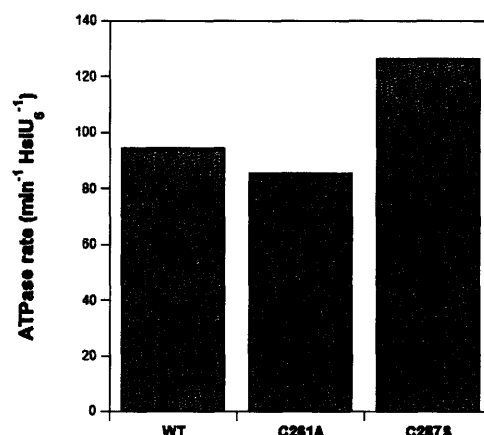


Figure 1. Substitutions for C261 or C287 in *E. coli* HslU result in mutant enzymes with ATP-hydrolysis rates similar to the wild-type enzyme.

When Cys¹⁵⁹ in *E. coli* HslV was replaced with serine or alanine, the mutant proteins were reported to be inactive in basal and HslU-activated peptidase cleavage (Yoo et al., 1998). Although Cys¹⁵⁹ in HslV is highly conserved in orthologs, aspartic acid was the next most common residue at this position, suggesting that a small side chain capable of carrying a negative charge might be important. To test this idea, I constructed and purified the C160D mutant of HslV. HslU stimulated Z-GGL-AMC cleavage by wild-type HslV and the mutant C160D enzyme to comparable extents, whereas significant activity was not observed in the absence of wild-type or mutant HslV (Fig. 2).

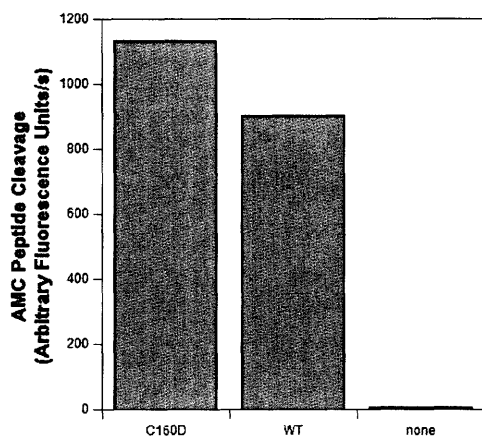


Figure 2. The C160D mutant and wild-type HslV (15 nM HslV₁₂) cleave Z-GGL-AMC at comparable rates in the presence of HslU₆ (50 nM). Significant cleavage was not observed with HslU₆ alone (right bar).

Next, I introduced cysteines in place of Gln³³³ or Ser³³⁸ in HslU to generate the C261A/C287S/S338C or C261A/C287S/Q333C mutants. In crystal structures, the side chains of Gln³³³ and Ser³³⁸ are exposed on the surface of an α -helix that is near the HslU-HslV interface. I also inserted a cysteine prior to the C-terminal His₆ tag of HslV to generate the C160D/Cys-H₆ mutant. As shown in Figure 3, mixtures of the HslU C261A/C287S/S338C and HslV C160D/Cys-H₆ mutant supported hydrolysis of Z-GGL-AMC at approximately 60% of the wild-type level. The apparent affinity constant for binding of HslU C261A/C287S/S338C and HslV C160D/Cys-H₆ was 34 nM. Similar results were obtained in experiments using mixtures of HslU C261A/C287S/Q333C and HslV C160D/Cys-H₆ (data not shown).

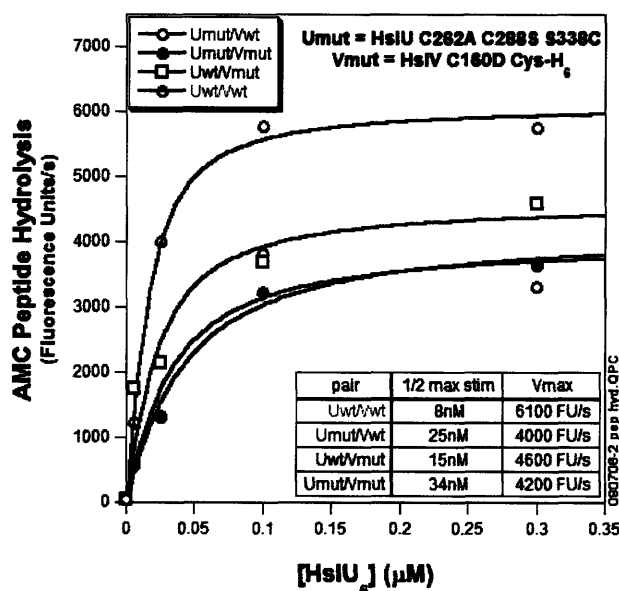


Figure 3. Peptidase hydrolysis by mixed complexes of HslU C261A/C287S/S338C and HslV C160D/Cys-H₆. Unlabelled cysteine mutants show approximately 60% wild-type activity.

HslU C261A/C287S/S338C, C261A/C287S/Q333C, and HslV C160D/Cys-H₆ were labeled with maleimide derivatives of fluorescein (FL), tetramethylrhodamine (TMR), Alexa 546 (A546), or QSY7. In several cases, proteins (especially HslV C160D/Cys-H₆) precipitated within several minutes of addition of the dye. Buffer controls showed that it was the modification by the dye, and not addition of the vehicle (DMSO), that caused precipitation. HslV C160D/Cys-H₆ precipitated following modification by every dye except FL. HslU C261A/C287S/S338C and C261A/C287S/Q333C precipitated when labeled with QSY7 but remained soluble when labeled with the other fluorophores.

Protein activity and the degree of labeling with the fluorescent dyes were inversely correlated. Following labeling reactions that proceeded to near completion, I found that

different fluorophore-labeled HslU-HslV pairs had 10-25% of wild-type activity in peptidase assays. I did not, however, observe significant changes in fluorescence or FRET signals when I mixed appropriate donor-labeled and acceptor-labeled HslU and HslV molecules in the presence of ATP γ S. Mixing incompletely labeled HslU or HslV molecules, which supported higher levels of peptidase activity, also failed to show any FRET changes. Based on HslUV crystal structures, the distance between some acceptor and donor dyes in fully labeled complexes should have been within the Förster distance (\approx 42-61 Å depending on the dye pair). I assume, therefore, that a potentially complicated set of interactions between the six HslU fluorophores and twelve HslV fluorophores prevents detectable FRET signal changes. When I used HslU and HslV molecules with sub saturating fluorophores, there was no way to control or assay for the possibility that acceptors and donors in HslUV complexes were close enough for efficient energy transfer.

Materials and Methods

HslU and HslV mutants were purified as previously described (Burton et al., 2005). Activity assays were conducted as described in chapter 2. Cysteine mutants of HslU₆ (8 μ M) and HslV (4 μ M) were labeled by overnight incubation with appropriate dyes at 4 °C in 100 mM Tris-HCl (pH 7.5), 125 mM NaCl. Unreacted dye was removed by four buffer exchanges using Micro-Bio Spin 6 chromatography columns (Bio-Rad Laboratories, Hercules, California). Maleimide derivatives of fluorescein, tetramethylrhodamine, Alexa 546, and QSY7 were obtained from Invitrogen (Carlsbad,

California) and were typically used in 25- to 100-fold excess over total cysteines in labeling reactions.

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